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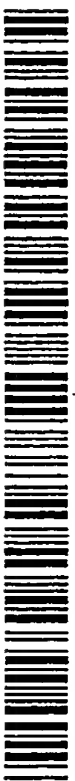
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(54) Title: **DEVICES AND METHODS FOR ASSESSING CELLULAR TRANSPORT FOR RESEARCH, DRUG DISCOVERY AND TESTING, CLINICAL DIAGNOSES, AND THERAPY DESIGN**

(57) Abstract: A method of measuring efflux/influx of a chemical drug (26), such as doxorubicin from a cell (14, 52), such as a human cancer cell or other biological cell, includes introducing the drug to the cell, for example by immersing the cell in a culture medium containing the drug or by direct injection into the cell. The cell containing the drug is then immersed in a drug-free liquid medium (28) and the efflux/influx of the drug from the cell measured with electrochemical equipment (60). For detection of efflux from small populations of cells, or a single cell, the signal strength may be increased by preconcentrating the drug on a working electrode (20, 50) and by increasing the oxygen concentration of the medium around the cell. A cell or small group of cells (52) to be studied may be spatially positioned on a substrate (58) by coating a small area (72) of the substrate with a layer (73) of a hydrophilic material to which the cell attaches.

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**DEVICES AND METHODS FOR ASSESSING CELLULAR  
TRANSPORT FOR RESEARCH, DRUG DISCOVERY  
AND TESTING, CLINICAL DIAGNOSES, AND THERAPY DESIGN**

**Background of the Invention**

The present invention is directed to the medical arts. It finds particular application in the detection of drug efflux from single or small populations of cancer cells and the assessment of drug resistance developed by the cells. It should be appreciated, however, that the method is also applicable to the detection of the transport of a variety of  
5 electroactive and/or optically active, or labelable drugs and other molecules including biomolecules inside cells, between intracellular compartments, through the walls of cells, between cells, in cellular architectures, and for solid tissue preparations.

One of the least well understood problems in cancer chemotherapy is the eventual resistance of tumor cells to different chemotherapeutic drugs of natural product origin,  
10 such as doxorubicin, actinomycin D, vinblastine, vincristine, or colchicine. Increasing the concentrations of one or more of these agents in small consecutive steps results in high-level cross-resistance in such cells to these as well as to many other, chemically unrelated drugs, particularly those which are hydrophobic and positively charged (i.e., weak bases) at physiological pH. Multidrug resistance (MDR) is a general term used for the process  
15 of cancer cells evading the cytotoxic effects of anticancer drugs. MDR is one of the major obstacles to successful chemotherapy of cancer.

The phenomenon of MDR can be attributed to several different biophysical processes: decrease in drug uptake, increase in drug efflux from resistant cells (outflow of drugs from the cells) leading to decreased intracellular drug accumulation and retention,  
20 increase in drug metabolism rate, or alterations in drug-target properties. The consequences of MDR are often catastrophic for the patient. The survival rate for cancer patients developing MDR is extremely low.

An MDR phenotype, which is characterized by decreased rates of accumulation, altered intracellular retention and distribution, and increased rates of efflux of drugs (and  
25 a variety of other cytotoxic compounds), may result from structural or functional changes of the plasma membrane, cellular compartments, or nucleus. Increased efflux has been

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found to correlate with the overexpression of the cell surface 170-kDa P-glycoprotein (Pgp) in many cancers, which utilizes the energy of ATP hydrolysis to actively exclude cytotoxic drugs against their concentration gradient, thus increasing efflux, whereas uptake is mainly due to passive diffusion via the plasma membrane. The underlying mechanisms whereby P-gp overexpression leads to MDR are still unclear. One hypothesis is that the P-gp belongs to the superfamily of ATP-dependent transporters and can utilize ATP to pump drug molecules out of the cells directly. It is unclear, however, how one transporter can extrude a variety of structurally different drugs. Another hypothesis is that most anticancer drugs are positively charged weak bases and P-gps can change the intracellular pH and membrane potential such that the drug molecules may be indirectly forced out of the cells through a partitioning equilibrium.

To understand the mechanisms of MDR, experimental methods are needed to study the dynamic characteristics of drug efflux from sensitive as well as drug-resistant cancer cells.

Several approaches have been used to measure drug efflux, including methods involving radio labeled drug extraction, flow cytometry, flow-through detection in a culture medium, and fluorescence. Even though all of these approaches have been successfully used for efflux studies on cell populations, each has its drawbacks. The first approach requires complicated, lengthy procedures (often more than 5 minutes to obtain one data point) including incubation, centrifugation, and drug extraction, followed by chromatographic or fluorescent and radioactive methods for detection. The cells are destroyed during the procedure, so only one efflux data point for a given efflux period can be obtained from each cell population.

Fluorescence detection schemes (flow cytometry, flow-through systems, fluorescence detection from single cells) can provide a relatively high temporal resolution (1-2 min/measurement). They are, however, generally imprecise due to weak fluorescence intensities, especially at low fluorescent drug concentrations. For fluorescence detection from single cells, the quenching effects due to drug binding to DNA and uneven drug distribution in the cytoplasm and plasma membrane are ignored, which renders the results inaccurate. The technique is suited to quantifying the efflux of many fluorescent drugs in cell population studies. However, flow cytometry becomes useless when the studied drug is nonfluorescent or binding to DNA and other target molecules, thereby quenching its fluorescence. Moreover, intracellular pH can affect drug fluorescence, leading to

erroneous data. Further, because the cells flow in this method, suspended cells are required.

In flow-through detection schemes, a monolayer of cells is attached to the bottom of a chamber and interacts with a drug-containing medium flowing over the cells. Fluorescence or absorbance of the studied drug in the medium obtained at the outlet of the system is measured and compared to the equivalent measurement in the inlet medium. The change in signal is related to drug uptake or efflux. This technique is designed for cell population studies, and an optical detection scheme is generally used in the flow-through system. A fluorescent drug is required when low drug concentrations are encountered.

These approaches generally rely on the use of a relatively large population of cells. With the exception of fluorescence detection from single cells, all conventional approaches can only be used to obtain drug transport data from entire cell populations, which makes it impossible to assess the heterogeneous behavior of individual cells. Assumptions have to be made based on average measurements. However, cell populations are not homogeneous. Based on the reported experimental approaches, continuous measurement of efflux of nonfluorescent drugs on the same cell culture in situ or assessing of efflux from a few cells or even a single cell is beyond the capabilities of existing technologies.

Another problem with using large populations of cells is that the effects of one cell may mask the reactions of an adjacent cell. For example, the efflux from one cell may overlap that of another, resulting in inaccurate cell efflux measurements.

Moreover, conventional methods do not allow observation of cell efflux and uptake at the same time. The sensors used are not able to detect whether the drug being detected has been absorbed by one or more cell prior to detection.

For studying multidrug resistance, information with high temporal resolution is critical to determine not only steady state but also initial influx and efflux rates. Single-cell resolution is desirable for modeling pump kinetics, since the local drug concentration is what the pump really senses, and bulk extracellular concentrations can be quite different from local levels adjacent to individual cells. These problems cannot be addressed with the techniques mentioned above: not even single-cell fluorescence measurement can assess extracellular drug concentrations directly at the studied cell.

Different carbon fiber microdisk electrodes have been used as sensors for real-time detection of exocytosis from single cells in neuroscience and endocrine secretion studies. The exocytotic release of catecholamines in vitro from single bovine adrenal medullary cells has been studied and the quantal nature of neurotransmitter exocytosis shown. By

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combining electrical capacitance measurement with microvoltammetry,  $\text{Ca}^{2+}$ -triggered exocytosis of catecholamines was studied in single neuroendocrine cells. The effect of autoreceptors on stimulated release of catecholamines from the cell body of a fully developed snail neuron were also studied *in vivo*. Insulin secretion from individual  
5 pancreatic  $\beta$ -cells has been carried out using carbon fiber microelectrodes. Some common anticancer drugs, such as daunorubicin and doxorubicin, besides being fluorescent, are also electroactive. Electrochemical methods could therefore be used to detect drug efflux from cancer cells.

Doxorubicin (also called Adriamycin), and some other anticancer drugs are  
10 "electroactive", i.e., they can be electrochemically reduced or oxidized, and are thus detectable by electrochemical methods. Electrochemical systems with a mercury electrode or a carbon paste electrode have been used to detect doxorubicin in a phosphate buffer and in patients' urine. The detrimental effect of mercury on live cells or the requirement of polishing the surface of a carbon paste electrode before each measurement, however, has,  
15 until recently, prevented these sensing schemes from being used for continuous cellular studies.

Recently, a cylindrical carbon fiber microelectrode has been used to monitor doxorubicin efflux from AUXB1 and  $\text{CH}^{\text{R}}\text{C5}$  cell monolayers. (These two cells lines are both from Chinese hamster ovarian cancer; AUXB1 is the parental cell line that is  
20 sensitive to anticancer drugs, whereas  $\text{CH}^{\text{R}}\text{C5}$  is the derived MDR subline that is drug resistant.) Both cathodic and anodic peaks (at  $\sim -630$  and  $250$  mV, respectively, vs  $\text{Ag}/\text{AgCl}$  at slow scan rates) are available for voltammetric measurements, but the electrodes were found to be more stable and the signal was larger when doxorubicin reduction was used. Due to strong adsorption of the drug molecules onto the electrode  
25 surface, adsorption preconcentration could be employed to enhance the signal, i.e., the respective adsorption peak. By applying special electrochemical pretreatment, cleaning, and preconcentration protocols, doxorubicin concentrations down to  $0.1 \mu\text{M}$  (in buffer with no live cells) could be detected.

The present invention provides for a new and improved apparatus and method for  
30 detection of electroactive drugs which overcomes the above-referenced problems, and others.

### Summary of the Invention

In accordance with one aspect of the present invention, a method of measuring efflux of a chemical from a cell or population of cells is provided. The method includes introducing the chemical to the cell and measuring an electrochemical property of a medium surrounding the cell with an electrochemical system which includes a working electrode and a reference electrode. The property is related to the concentration of the chemical in the medium. The method is characterized by adding oxygen to the medium to increase a signal strength of the electrochemical property.

In accordance with another aspect of the present invention, an apparatus for measuring efflux of a chemical from a biological cell or population of cells is provided. The apparatus includes a substrate having a surface which receives the cell, and a medium on the substrate. An electrochemical monitoring system measures an electrochemical property of the medium surrounding the cell, the property being related to a concentration of the chemical in the medium. The apparatus is characterized by the substrate surface having at least one attachment region to which the cell or population of cells attaches, which is surrounded by a region which resists the attachment of cells.

In accordance with another aspect of the present invention, a method of measuring efflux of a chemical from a biological cell or a cell population is provided. The method includes introducing the chemical to the cell or cells and measuring a property of a medium surrounding the cell or cells. The property is related to a concentration of the chemical in the medium. The method is characterized by positioning the cell or cell population on a surface of a substrate by attachment of the cell population to a region of the substrate which permits attachment, the attachment region of the substrate being surrounded by a region which resists attachment of cells.

In accordance with another aspect of the present invention, a method of measuring transport of a chemical across a membrane of a human or other biological cell is provided. The method includes exposing the cell to the chemical and measuring a property of a liquid medium disposed outside the cell. The property is related to a concentration of the chemical in the medium. The method is characterized by providing a substrate surface with a region formed from a material to which the cell attaches, the region being surrounded by a portion of the surface which resists attachment of a cell and patterning the substrate using photolithographic techniques to define at least one sensor adjacent the attachment region for sensing the property of the liquid medium. The method further includes depositing the cell on the attachment region and, after the step of exposing the

cell to the chemical, detecting the property of the liquid medium surrounding the cell and determining the concentration of the chemical in the medium therefrom.

In accordance with another aspect of the present invention, a method of measuring transport of a chemical across a membrane of a biological cell is provided. The method includes exposing the cell to the chemical and providing a substrate surface with a site formed from a material to which the cell attaches, the attachment region being surrounded by a portion of the surface which resists attachment of a cell. The method further includes depositing the cell on the site and moving a sensor through a wall of the cell to contact the material in the cell. Further, the method includes measuring a property of a material within the cell with the sensor, the property being related to a concentration of the chemical in the cell with the sensor and determining the concentration of the chemical in the cell therefrom.

One advantage of the present invention is the provision of a method of detecting drug efflux from cancerous cells.

Another advantage of the present invention is that the method is suitable to single cell study.

Yet another advantage of the present invention is that the method provides real time monitoring of cell activity in a population of cells or from a single cell.

A further advantage of the present invention is that it permits the study of continuous single-cell-level drug efflux.

A still further advantage of the present invention is the provision of a method of distinguishing between drug-resistant and drug-sensitive cells.

A yet further advantage of the present invention is the provision of in-situ monitoring of drugs which may be nonfluorescent and optically inactive in general.

Another advantage of the present invention is that it enables the detection of other molecules, such as pharmacological agents and biomolecules, such as neurotransmitters.

Another advantage of the present invention is the provision of a system in which a liquid medium is maintained above and below biological cells being investigated.

A yet still further advantage of the present invention derives from the ability to study arrays of electrochemical cells, each one obtaining information from a single cell or a population of cells.

Still further advantages of the present invention will become apparent to those of ordinary skill in the art upon reading and understanding the following detailed description of the preferred embodiments.



### Brief Description of the Drawings

The invention may take form in various components and arrangements of components, and in various steps and arrangements of steps. The drawings are only for purposes of illustrating a preferred embodiment and are not to be construed as limiting the invention.

FIGURE 1 is a side view of a system for assessing efflux with a carbon fiber microdisk electrode (CFDE) positioned approximately  $1\mu$  away from a single cell by a 3 axis hydraulic micromanipulator, according to the present invention;

FIGURE 2 is an enlarged view of the tip of the CFDE;

FIGURE 3 is a side view of a second embodiment of a system for assessing efflux with a carbon ring electrode, according to the present invention;

FIGURE 4 is a top view of the embodiment of FIGURE 3;

FIGURE 5 shows the chemical structure of a doxorubicin molecule;

FIGURE 6 is a top view of a third embodiment of a system for assessing efflux from spatially orientated cells with a carbon ring electrode, according to the present invention;

FIGURE 7 is a top view of a fourth embodiment of an array system for simultaneous assessment of efflux from cells with a plurality of carbon ring electrodes, according to the present invention;

FIGURE 8 is a top view of a fifth embodiment for assessing efflux from cells with a carbon electrode, pH and calcium monitors and sensors for other ions, according to the present invention;

FIGURE 9 is a side sectional view of a sixth embodiment of a system for injection of a chemical into a cell and assessing efflux therefrom with a carbon ring electrode or other sensors, according to the present invention;

FIGURE 10 is a side sectional view of the embodiment of FIGURE 9, during drug injection;

FIGURE 11 is a side sectional view of a system suited to the study of cell monolayers or closely spaced cells, according to the present invention;

FIGURE 12 is a top view of the system of FIGURE 11;

FIGURE 13 is a side sectional view of a system suited to measuring chemical transfer across gap junctions between cells, according to the present invention;

FIGURE 14 is a side sectional view of a system for studying variations across a mass of cells, according to the present invention;



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FIGURE 15 is a plot showing the dynamics of doxorubicin adsorption onto a stabilized CFDE surface in oxygen-free efflux medium containing 1  $\mu$ M doxorubicin;

FIGURE 16 is a calibration curve showing the output of the detecting electrode versus doxorubicin concentrations (the output is in the form of differential current as  
5 obtained with square wave voltammetry scans);

FIGURE 17 is a plot showing the catalytic effect of oxygen on doxorubicin detection for the cathodic scheme (negative going scan);

FIGURE 18 is a plot showing the catalytic effect of oxygen on doxorubicin detection for the anodic scheme (positive going scan);

10 FIGURE 19 is a plot showing individual OSWV sweeps at different efflux times for drug efflux from a single sensitive (AUXB1) cell in efflux medium;

FIGURE 20 is a plot showing individual OSWV sweeps at different efflux times for drug efflux from a single drug-resistant ( $CH^R C5$ ) cell in efflux medium;

FIGURE 21 is a plot showing doxorubicin concentrations for the single cells of  
15 FIGURES 19 and 20, over time; and,

FIGURE 22 is a calibration of a CFDE for doxorubicin, plotting the peak height,  $\Delta i_p/\Delta E$ , of the cathodic OSWV peaks at -650 mV versus Ag|AgCl in efflux medium against a logarithmic concentration axis.

## 20 Detailed Description of the Preferred Embodiments

Simple and fast detection schemes are realized by using the electroactive properties of drugs, such as the cancer treating drugs doxorubicin, actinomycin D, vinblastine, vincristine, and colchicine. While particular reference is made herein to the detection of doxorubicin, it should be appreciated that the methods are also applicable to the detection  
25 of other electroactive drugs, chemicals, and ions (all generally referred to herein as chemicals). By way of example, cells from two cell lines, AUXB1 and  $CH^R C5$ , may be preloaded with doxorubicin, and their drug efflux properties monitored using a working electrode, such as a carbon fiber microdisk electrode (CFDE) to measure local instantaneous concentrations of the effluxed drug molecules at single cells, spatially  
30 arranged populations of cells, cell monolayers, and sections of tumors comprising populations of closely spaced cells. Human, other animal, and plant cell(s) can be studied with the techniques described.

While particular reference is made to the detection of the transport of chemicals (including ions) across a cell membrane by electrochemical techniques, it is to be

appreciated that the techniques described for the study of transport in single cells and populations of cells are also applicable to other detection methods, including, but not limited to fluorescence, pH detection, ion transport detection (such as  $\text{Ca}^{2+}$ ,  $\text{K}^{+}$ , and the like), and oxygen sensing. In some cases, metabolites of the chemicals, rather than the  
5 chemicals themselves, are detected.

Application areas include research, drug discovery and testing, clinical diagnoses, and therapy design, among others. The cell types covered include cancer cells (drug sensitive as well as multidrug resistant ones), epithelial and other monolayers of polarized cells and such tissues. Nerve cells are another example: neurotransmitter release from  
10 individual nerve cells can be monitored. For example, with a matrix of many little sensors even spatial distribution of release can be monitored; nerve cell architectures can also be made, with synapses monitored with many small neurotransmitter sensors positioned around the cells to assess firing events and their spatial effects.

One preferred electrochemical method for measuring drug or other chemical efflux  
15 from a cell or cells, such as a tumor cell, involves the following steps, which need not be carried out in the given order:

1. Preparing the working electrode -electrode cleaning and/or preconditioning.
2. Preparing the cells to be studied:  
20           a) charging (e.g. loading, incubating) cells with drugs/other chemicals.  
           b) washing cells to remove drugs/other chemicals on the cell exterior.  
           c) positioning cell(s) on a substrate.  
25           d) immersing cells in test (efflux) medium.
3. Positioning electrode(s) in test medium with cells.
4. Monitoring efflux from the cell(s) using electrochemical monitoring equipment:  
30           a) preconcentration of effluxed drug at the electrode surface.  
           b) measuring signals corresponding to drug concentration.  
           c) comparing signals to calibration curves to determine efflux concentration.  
           d) regeneration of electrode surface.

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e) repeat of steps a)-d) for obtaining additional data points over time.

The detection methods are not limited to drug (or other chemical) efflux, but can also be used for studying other transport mechanisms, including chemical influx. For example the movement of a chemical into a cell can be studied by immersing a cell in a culture medium containing the chemical and studying the increase in the chemical within the cell, as will be described in greater detail below.

Other methods for measuring efflux/influx can be used, including, but not limited to fluorescence measurements, radio labeled drug extraction, flow cytometry, flow-through detection in a culture medium, and the like.

With reference to FIGURES 1 and 2, an electrochemical system A for the detection of drug efflux from cells is shown. The system includes a shallow tray or efflux container 10. A single cell 14 or population of cells (such as a monolayer of cells or section of a tumor) to be tested is positioned on a planar substrate, such as the base 17 of the efflux container. An electrode system 18, including a working electrode, such as a carbon fiber microdisk electrode (CFDE) 20, and a reference electrode 24, are used to monitor the efflux of a selected electroactive drug 26, such as doxorubicin, from the cell(s), i.e., drugs which have passed through the cell membrane or cell wall 27.

The efflux container 10 may be made of plexiglass and may be covered by a plastic cover (not shown), which ensures free air exchange between inside and outside the covered space defined between the efflux container and the cover. An efflux medium 28 is contained within the efflux container 10. The reference electrode is positioned in or is in electrical contact with the medium 28. Suitable efflux media are those capable of maintaining viability of the cells, such as minimum essential medium (MEM). one suitable efflux medium comprises NaCl 140 mM, KCl 5.4 mM, Hepes 5.5 mM, CaCl<sub>2</sub> 2.5 mM, MgCl<sub>2</sub> 0.5 mM, and glucose 11 mM adjusted to pH 7.4 with 1 M NaOH. The detecting portions of the electrodes and the cell or cells are in, and covered by the efflux medium so that chemicals effluxing from the cell enter the medium. The depth of the medium is preferably about 1 mm, although greater or lesser depths of medium can be used, depending on whether a single cell or a population of cells is under study, and the type of electrodes used. The volume of efflux medium is defined by the size of the container and the depth of the efflux solution. This latter is typically kept low, so that free oxygen can easily diffuse down to the cell(s) studied (to avoid hypoxic conditions, unless desired). The surface area can be that of a Petri dish or a lot smaller. It is determined

really by the size of the preparation. For a single cell, the diameter of the container can be as low as a few hundred microns.

The CFDE 20 preferably comprises a carbon fiber core 30, surrounded by an electrode body or sheath 32, which may be formed from pulled glass and/or polyethylene tube. In the embodiment of FIGURES 1 and 2, a tapered portion 34 of the body, adjacent a tip 36 thereof is formed from polyethylene and a second portion 38 is formed from a glass capillary.

The CFDE may be fabricated by sucking a carbon fiber (approximately 4-5 cm in length) into a piece of polyethylene tubing, approximately 2-3 cm in length by applying a low vacuum, leaving about 1 cm of carbon fiber extending from both ends. The tubing is then heated above a hot soldering iron and pulled to form a thin section in the middle. After touching the soldering iron very briefly to this section, only a submicrometer-thick polyethylene insulation remains around the carbon fiber. The assembly is then cut in the middle with a scalpel. The polyethylene at the other end is attached to a glass capillary to form the electrode body 32. Part of the fiber is cut to the appropriate length to establish electrical contact between the carbon fiber 30 and the stripped end 40 of an insulated wire 42 dipped in mercury 44. The mercury in the glass capillary thus makes contact between the carbon fiber and the insulated wire. This fabrication procedure provides a tight enough seal between the carbon fiber 30 and the pulled tip of the glass capillary such that an adhesive sealant need not be applied inside the capillary tip 36.

The reference electrode 24 can be formed from a variety of materials. Particularly preferred is a reference electrode of silver/silver chloride.

It will be appreciated that a variety of other electrode systems 18 may alternatively be utilized. For example, a system having a counter electrode (which may be formed, for example, from stainless steel) in addition to the working and reference electrodes may be employed. Other materials or configurations may also be used for the working and reference electrodes. For example, a ring electrode 50, such as a carbon ring, which surrounds a cell 52 or population of cells may be used in combination with a reference electrode 54, as shown in FIGURES 3 and 4. In the system of FIGURES 3 and 4, in place of an efflux container, an annular ring of material 56 is optionally used to contain the efflux medium 28 on a planar support plate 58 or other substrate.

With reference also to FIGURES 1 and 2, the electrodes 20 and 24 (and likewise 50, 54) are connected with electrochemical monitoring equipment 60. As shown schematically in FIGURE 1, the monitoring equipment 60 preferably includes a computer

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controlled potentiostat 62 and a computer 64 for data storage. The electrodes and monitoring equipment thus comprise an electrochemical system for detection of electrical signals generated between the electrodes as voltages and/or currents are applied to the electrodes. By selection of appropriate applied voltages/currents, the signals detected from the medium correlate with the concentration of the drug in the adjacent medium and therefore can be used to determine the concentration of drug, and the efflux of the drug from the cell or cells over time, into the medium.

Equipment suitable for efflux monitoring includes a working electrode (e.g. the described carbon microelectrodes 20, 50, a reference electrode 24, 54, and preferably a counter (or auxiliary) electrode (not shown), which is immersed in or in electrical contact with the efflux medium 28. All three are connected to a potentiostat and/or galvanostat. In cases when the overall current is very small, it is possible to use a macro reference as both the reference and the counter electrodes. In such cases, it is possible to use a simple two electrode system without the need for a third electrode. Such a two electrode system may include a high speed - low current amplifier in series with the two electrodes and a DA port of a computer that generates the input voltage waveform (in place of a potentiostat and/or galvanostat). The current response is then recorded and analyzed by the controlling computer. For multiple electrode-based recordings (such as systems for the study of several simultaneously effluxing cells, or several sensors around one cell), a multichannel version of the above equipment may be used. However, it is possible also to use a multiplexer to input all the monitored variables into the computer sequentially and periodically (since the signals are typically slow here compared to the speed of even average data acquisition rates). Driving the different sensors is feasible by demultiplexing DA outputs from the same computer, especially when two-electrode arrangements are used. Sample-and-hold circuits are then preferably used to keep the output values constant for the different individuals sensors and devices until the next DA values are sent to them.

The working electrode 20, 50 is positioned in the efflux medium 28 a selected distance from the cell 14, the distance preferably in the range of about 0.5- 2  $\mu$ . In the embodiment of FIGURE 1, a micromanipulator 65, such as a fine three-axis hydraulic micromanipulator (MO-203, Narishige, Tokyo, Japan) mounted on a Diaphot microscope, can be used to position the electrode adjacent the cell(s). To monitor efflux from a single cancer cell 14, the tip of a stabilized CFDE 20 is moved by the micromanipulator to touch a suitable cell slightly and then withdrawn to about 1  $\mu$ m away from the cell. Other methods of positioning the electrode relative to the cell are discussed below.

Optionally, as shown in FIGURE 4, a stirring system 66, such as a micromechanical motor (MEM), which may be positioned on the substrate in an annular ring around the cell, stirs the efflux medium during measurements.

FIGURE 5 shows a doxorubicin molecule.

5 To study the efflux of the selected drug from the selected cell(s) 14, cultured cells are exposed to the drug and then the efflux of the drug from the cells is detected in an efflux medium 28 which is initially free of the drug. Electrochemical monitoring methods are preferably used for these efflux measurements, the drug concentration being determined from electrical signals received from the electrodes using a calibration curve.  
10 Between efflux measurements, the electrode is electrochemically preconditioned to regenerate the electrode surface and a preconcentration step is preferably carried out to preconcentrate the drug at the electrode surface to enhance the strength of the signal.

A fresh (or regenerated) working electrode 20, 50 is preferably used for every efflux experiment. The electrode is preferably kept in situ throughout the experiment  
15 without any mechanical cleaning procedure between measurements, allowing measurements to be obtained at closely spaced time intervals.

For studies on single cells 14, the minimum distance between nearest-neighbor cells in both lines is preferably no less than 150  $\mu\text{m}$ , more preferably no less than 250  $\mu\text{m}$  to ensure that no (or substantially no) interference from efflux from nearby cells can affect  
20 the detected efflux signal of the studied single cell. However, for studies in which interactions between cells are to be measured, the cells are preferably closely spaced, i.e., no more than about 100  $\mu\text{m}$  apart, more preferably, less than 50  $\mu\text{m}$  apart. Optically, a gradual change in intercellular distance may be employed in such experiments, from about 250  $\mu\text{m}$  down to zero (= touching).

25 The cells to be used are preferably attached to a suitable substrate, such as a glass coverslip or other suitable material, such as a silicon sheet.

In one embodiment, the cells are grown on a coverslip (not shown) which is then placed on the base 17 of the efflux container 10. Specifically, the cells are first cultured on a coverslip in a culture medium prepared to the same formulation as is used for the  
30 efflux medium. The culture medium is then replaced by fresh culture medium and the drug added. The cells are then incubated in this drug-containing medium at a suitable temperature and for a sufficient time for the cell or cells to absorb the drug. Typically, for doxorubicin, about 1 hour at 37 °C is sufficient. After incubation of the cells with the drug, the coverslip with the monolayer 14 of drug loaded cells is washed to remove excess

extracellular drug, preferably in an ice-cold phosphate-buffered saline (PBS) solution, and placed immediately after the last washing step into the efflux measurement container 10 with the efflux medium. The efflux experiment is preferably carried out at a suitable temperature for normal activity of the cells, e.g., 37 °C .

5 In an alternative embodiment, a cell or a group of cells may be grown in a medium and then spatially positioned on a substrate after growth. By positioning the cell(s) relative to a fixed electrode it is not necessary to use complex positioning system to position the electrode in the desired proximity to the cell. With reference to FIGURES 3 and 4, the support plate 58 includes a substrate 70, such as a sheet or wafer of silicon  
10 material, Pyrex™ glass, or other inert materials suitable for a base for layer-by-layer deposition of microfabricated structures on top, which has one or more regions or sites 72 defined by a layer or area 73 which attracts the cells and allows them to attach, such as a hydrophilic material on a top surface 74 thereof. The hydrophilic material can be just relatively hydrophilic compared to the surroundings, such as surface-coated silicon, or  
15 silicon when the surroundings are coated with a more hydrophobic material, like polyimide. It is also contemplated depositing a layer of a cell adhesion promoter such as collagen, or pro-adhesion proteins on the substrate surface. The remainder of the top surface (or at least a region surrounding the attachment area 72) resists attachment, for example, hydrophobic material 76, such as silicon itself, Pyrex™, other glasses, or  
20 polyimide.

The medium containing the cells is placed on the substrate, for example, by placing a drop on the top surface. The cells to be tested are attracted to the hydrophilic region(s) 72 and settle out from the medium and attach to the hydrophilic regions.

After attachment, any remaining non-attached cells can be washed off the substrate  
25 by rinsing, e.g. with water or medium. In the embodiment of FIGURE 3, a single hydrophilic region 72 is used, which is in the shape of a circle, just large enough to accommodate a single cell 52 (its diameter depending on the type of cell used, typically in the order of 5-50  $\mu\text{m}$ ; non-circular areas are also possible for elongated cell types). The hydrophilic region is preferably centered in the middle of the carbon ring electrode 50, so  
30 that all regions of the electrode are approximately the same distance from the cell, and at a suitable distance for obtaining efflux measurement. The distance is preferably from zero to 1-2  $\mu\text{m}$ . However, when it is important to either protect the sensor from cellular contamination (e.g. by proteins) or to provide a more ideal geometry for concentration distribution, a larger distance may also be used, such as 5-10  $\mu\text{m}$ . The carbon ring



electrode may be positioned, for example, at the periphery of the hydrophilic region 72, or on the adjacent hydrophobic region 76. The efflux medium is retained by forming a well or other depression in the substrate, or by forming retaining walls on top of the substrate.

Alternatively, the region 72 may be sized to accommodate a number of cells, so that interactions of cells may be studied. Or, as shown in FIGURE 6, several hydrophilic regions 72' may be defined on a substrate in a selected spatial orientation, and surrounded by an electrode 52, which receives signals resulting from efflux from one or more cells on each of the regions. In the embodiment of FIGURE 6, seven such regions 72' are arranged in an annular ring, each one equidistant from the carbon ring electrode 52, although a variety of other spatial orientations and number of regions are also contemplated. In this way, the effects of the efflux of a drug from one cell on the efflux of spatially arranged neighboring cells can be studied.

In this embodiment, the drug and/or other chemicals may be introduced to the cells in the culture medium, or after attachment of the cell(s) to the substrate. In one specific embodiment, the drugs and/or other chemicals to be studied are introduced directly into the cells on the substrate, through the cell wall, as will be described in greater detail herein.

The systems shown in FIGURES 3-4 and 6-8 are readily formed, for example, by microfabrication techniques, such as photolithography, thin and thick film printing, and the like to lay down and pattern the attractive sites, electrodes, wiring, and other features on the substrate. This allows the systems to be very small, preferably only a few micrometers in diameter (e.g. 10-50 $\mu$ ), when a single cell is to be accommodated. Cell architectures of micrometer precision can thus be realized and tested for many variables simultaneously, or consecutively.

With reference now to FIGURE 7, the system is amenable to separate and simultaneous study of multiple cancer cells 14 or groups of cancer cells using an array 80 of electrochemical cells 82. FIGURE 7 shows nine such electrochemical cells 82, although it will be appreciated that large arrays of cells can be fabricated on a single substrate 84 using microfabrication techniques. The cells are each defined by a well 86, which contains a small droplet of efflux medium 28. Carbon electrodes 88 in each of the cells are hooked up to electrochemical monitoring system 90 by wiring 92 which passes through to the underside of the substrate (or which is deposited on the upper side, and then an insulating layer deposited on top). A common computer controlled monitoring system is preferably employed, which allows for simultaneous or sequential data collection and

data analysis for all of the electrochemical cells. As before, a single cell 14, or a population of cells, is positioned in each of the wells on a hydrophilic area 96. Alternatively, each of the biological cells or cell populations may be under the same medium, i.e. in one well, to see how these individual cells or "islets" of compact cells interact with each other. The sensor array is also good for monitoring spatial distribution around single cells (nerve) and other architectures.

### Preparation of the working electrode

To prepare the working electrode, it is cleaned, prior to the efflux experiment. Although mechanical cleaning methods may be used, electrochemical cleaning is conveniently performed. For example, the CFDE 20 (or other carbon electrode) may be stabilized in a separate container, prior to an efflux experiment, by running cyclic voltammetry (CV) from -200 to +600 mV at 100 mV/s twenty times in fresh efflux medium, and then conducting Osteryoung Square Wave Voltammetry (OSWV) from -300 mV to -800 mV at 60 mV/s, five times. This procedure was found to be effective for removing traces of doxorubicin from the electrode.

The prepared electrode may be stored in a container of the efflux medium for several hours prior to the experiment. Preferably, the temperature of the storage efflux medium is about the same as that to be used in efflux experiments.

Mechanical electrode cleaning is possible with commercial microelectrode polisher - bevellers, using a fine grain polishing material. Other cleaning methods include chemical handling, such as rinsing (or used after a longer period of cleaning) with chemicals in organic media. It is also contemplated using acetonitrile as a medium and isopropyl alcohol as a cleaner.

Other electrochemical cleaning methods include reductive (cathodic) or oxidative (anodic) treatments, periodic cleaning waveforms, and oscillating between extreme voltages. The parameters, frequency and duration of such methods depend on the nature of the substance to be removed and thus are preferably optimized empirically in a given type of study.

It is also important to clean (regenerate) the electrode between each measurement. This precleaning is important, particularly in the case of strongly absorbent molecules such as doxorubicin. A doxorubicin molecule has four adjacent and co-planar hexagonal rings (See FIGURE 5), which can match the benzene rings of the basal plane of graphite. The molecule was found to adsorb well onto a carbon electrode. Since doxorubicin contains

both a quinone and a hydroquinone functionality, as shown in FIGURE 5, it can be electrochemically reduced or oxidized.

The carbonyl side chain can also yield a reduction current at very negative potentials. Two characteristic peaks related to doxorubicin are obtained with 5 microvoltammetry after adsorptive preconcentration of doxorubicin on the carbon electrode surface. One is a cathodic peak at about -630 mV, and the other is an anodic peak around +250 mV. Both peaks are useful for analytical purposes. The positive potentials applied to obtain the anodic peak (especially those above +250 mV), however, tend to affect the surface of a carbon electrode by generating oxidized surface groups, 10 leading to insufficient sensitivity for efflux measurements after a period of time. Using the cathodic peak, a more sensitive and stable efflux measurement procedure can be realized because less damage is done by the measurement itself to the active sites on the surface of the carbon. Therefore, the cathodic peak at around -630 mV versus Ag/AgCl in PBS is preferably used for efflux measurements.

15 As a consequence of the strong absorption, it is highly desirable to clean (regenerate) the sensing surface of the electrode before each measurement, to remove adsorbed doxorubicin residues from the previous measurement and thus ensure identical initial conditions for adsorption for each data point. For real time monitoring, electrochemical cleaning is preferred. This may be carried out, for example, with pulses 20 of about -1000 mV. For carbon electrodes, a pulse length of from about 5-10 seconds, is generally sufficient to regenerate the working electrode when the concentration of the electroactive drug up to about 0.1  $\mu$ M. At higher concentrations, up to about 1-2  $\mu$ M of the drug, a regeneration time of about 10-20 seconds is preferred.

Drug adsorption can be used for preconcentration of doxorubicin on the working 25 electrode surface, thus amplifying the signal that might otherwise be too small to detect, due to typically low concentrations encountered in single cell efflux experiments.

Even though a diffusion peak becomes also apparent in CV or OSWV in both anodic and cathodic scans at very high doxorubicin concentrations (in the order of 100  $\mu$ M, not shown), in the practical concentration range of efflux experiments, only 30 adsorption peaks can be discerned. The height (or area) of these peaks can be used to study the dynamics of adsorption onto a working electrode, such as a CFDE.

Preconcentration may be carried out as follows. Prior to each measurement, the effluxing drug is allowed to preconcentrate on the working electrode surface for a period of time sufficient for a substantially stable state to be achieved. For doxorubicin, a

preconcentration period of about 20 seconds, or more, for drug concentrations within the 0.1 – 1.5  $\mu\text{M}$  range is preferred. 20 seconds corresponds to about 90% completion of the adsorption process. Also, small errors in this period will not affect significantly the results, due to the relatively small slope of the dynamic saturation curve at times of about 5 20 seconds, and above.

Preconcentration may involve just a waiting period with open circuiting. However, a polarized electrode is sometimes preferred for preconcentrating charged particles (like certain drugs). Alternatively, methods involving a current pass during preconcentration are used, particularly, when the species that adsorbs is an electro-reduced or oxidized 10 version of the substance to be preconcentrated.

Since adsorption currents are proportional to scan rate, adsorption can also be used to amplify the signals obtained during efflux experiments. Relatively high scan rates (about 800 mV/s) are preferred to provide high temporal resolution for adsorption kinetics studies. The higher scan rate, in turn, rationalizes the negative shift of the peaks with 15 respect to the -650 mV usually observed.

Measurement of the concentration of doxorubicin, or other effluxing drug or other chemical, may be carried out by a variety of electrochemical techniques, such as differential pulse voltammetry (DVP), square wave voltammetry (e.g., Osteryoung Square Wave Voltammetry- OSWV) , or other suitable measuring techniques.

20 In DVP, the potentiostat applies electrical potentials and detects signals which are transferred to the computer for data storage. Specifically, for doxorubicin, a DPV scan is preferably performed from -300 to -1000 mV, using a scan rate of about 20mV/sec, a pulse amplitude of about 50 mV, a pulse width of about 50 ms and a pulse period of about 200 ms. Other potential scan ranges may be used for other drugs, depending on the potential 25 at which oxidation or reduction of the drug occurs.

In OSWV, Doxorubicin detection using its cathodic peak may be performed by scanning from about -300 mV to -800 mV at about 60 mV/s. Or, anodic detection may be performed, for example, using OSWV scans from 200 to 600 mV at 60 mV/s .

In cathodic OSWV, the voltage of the working electrode 20 is measured with 30 respect to the Ag/AgCl reference electrode and the differential current ( $\Delta i/\Delta E$ ) measured over the range of applied potentials in order to determine the peak height of the differential current ( $\Delta i/\Delta E$ ), which occurs at around -630 mV vs Ag/AgCl for the cathodic peak of doxorubicin. The signals received from the electrodes 20, 50, 24 are subsequently converted by the computer to concentration measurements, using a calibration curve, as

will be described below. Because differential current rather than current is recorded,  $\Delta i/\Delta E$  values are employed for calculating concentrations. Measurements are repeated at intervals, preferably around 0.5-10 minutes between measurements, more preferably, about 1 minute between measurements for OSWV. This gives time for the electrode to be  
5 thoroughly regenerated and for preconcentration to take place between each measurement.

Once sufficient measurements have been taken, the microelectrode 20, 50 is then calibrated in a series of prepared solutions comprising the efflux medium and known concentrations of the drug. Preferably, at least two concentrations are used, representing the upper and lower ends of the range of expected concentrations in the efflux  
10 measurements. The temperature of the efflux medium during calibration is preferably the same as that used for the efflux measurements, i.e., about 37°C for doxorubicin efflux from live cells. The same measurement protocol as the one used during the efflux experiment is applied to the microelectrode 20, 50.

The height of peak  $\Delta i_p/\Delta E$  for the microelectrode has been found to be a log linear  
15 function of the doxorubicin molar concentration over a broad range of concentrations. This may be due to the logarithmic character of the adsorption isotherm of doxorubicin on carbon within the concentration range studied. The values of the peak heights from the actual efflux measurements are then transformed to corresponding drug concentrations according to this two (or more)-point calibration.

20 Because most deactivation of the working electrode 20, 50 occurs during the first period of electrode stabilization and very little sensitivity loss occurs later, postcalibration can be used for the entire period of efflux measurement, without the need for extra corrections.

The calibration of the working electrode 20, 50 is preferably carried out  
25 immediately after each drug efflux experiment using test solutions encompassing the same range as is measured in the efflux experiment, such as in the 0.1 to 1.5  $\mu\text{M}$  range. The temperature of the efflux medium during calibration is preferably the same as that used for the efflux experiments, e.g., 37°C, and the measurement protocol is the same as for the efflux measurements.

30 For example, the microelectrode 20, 50 used for the efflux measurements is calibrated after each efflux experiment in fresh efflux medium containing known concentrations of the drug. Peak heights are determined graphically and plotted against a logarithmic concentration axis. The peak heights determine a linear calibration to be used with respect to the logarithm of drug concentration. The values of the peak heights

from the actual efflux measurement are then transformed to corresponding drug concentrations according to the calibration graph.

Other methods of detection are also contemplated, including the use of waveforms other than square wave, and evaluation of the signal, for example, peak area, integral scans, and differential scans. Instead of a differential current, the measuring technique may use features of a single scan (especially in adsorption current cases). Different types of amperometry and differential amperometry may be used for detecting faster events (such as quantile release of drugs and other substances) where scanning is not sufficiently fast to register the events or not necessary, such as in stable processes that can be separated easily from those produced by other substances present.

It has been found, unexpectedly, that an increase in signal strength can be obtained by raising the oxygen concentration of the efflux medium during efflux measurements above the normal oxygen concentration of the medium. A variety of methods can be used for increasing the oxygen concentration. For example a stream of oxygen, or a gas which is enriched with oxygen, can be bubbled into the efflux medium to increase the oxygen content of the medium. As shown in FIGURE 1, a tube 100 supplies the efflux medium with an oxygen rich gas (i.e., preferably more than 21% oxygen) from a source of the gas 102, such as a pressurized oxygen container. Other methods of enriching the medium with oxygen are also contemplated.

The effect of oxygen concentration was found to be higher at 100% oxygen (pure oxygen) than for air (21% oxygen) thus, it is preferably to use a gas with more than 21% oxygen, more preferably, at least 50% oxygen, and most preferably, over 90% oxygen. An amplification factor of 20-25 was found in 100% oxygen for cathodic scans using doxorubicin. Anodic scans for doxorubicin have not been found to show an improvement in signal strength with increasing oxygen, thus, the combination of increased oxygen and a microvoltammetry method using a cathodic scan is preferred.

An electrocatalytic effect due to dissolved oxygen may be the reason for the increases in signal strength observed: the doxorubicin molecules just reduced can be oxidized back by dissolved oxygen and thus recycled to produce further reduction current.

Although a small amplification effect has been previously shown in the anodic determination of dopamine (DA) in the presence of ascorbic acid (AA) using a carbon paste working electrode using amperometry, only an insignificant signal enhancement (3×) was obtained using carbon electrodes. This was rationalized by the radial diffusion patterns around a CFDE, leading to efficient mass transport both to and from the electrode



which reduces the likelihood of ascorbate-produced dopamine molecules returning to the electrode, and yielding further oxidation current.

Contrary to these results, a 5-6× and 20-25× signal enhancement for doxorubicin reduction at CFDEs in the presence of 21% and 100% oxygen, respectively, has now been shown for drug efflux from cells, as compared with an oxygen free (e.g. pure nitrogen-containing medium). Since the diffusivities of dopamine and doxorubicin do not differ much ( $5.0 \times 10^{-6}$  cm<sup>2</sup>/s for dopamine and  $1.5 \times 10^{-6}$  cm<sup>2</sup>/s for doxorubicin), these results suggest that chemical re-oxidation of the electro-reduced doxorubicin molecules by oxygen occurs when the reduced product molecules are still “trapped” at the electrode, i.e., in their surface adsorbed state. This suggests why the scheme using reduction can provide a much lower detection limit. Thus, single cell efflux measurements are preferably carried out with the cathodic scheme.

The working electrode may be microfabricated from graphitic carbon, as well as other types of carbon in the form of microelectrode rings, arches, dots or the like. Suitable electrode materials include diamond-like carbon, doped diamond-like carbon (to enhance conductivity), and nitrogen incorporated carbon, among others. When ionic concentrations are to be measured, suitable ion selective microelectrodes as known in the art are used. For example, Pd/PdO can be used for measuring pH, or Pt for oxygen measurements.

## 20 Preparation of Cells for Cell Efflux Experiments

The cells may be loaded with the drug, prior to efflux experiments, by absorption from a medium containing the drug (incubation), by direct injection of the drug into the cell(s), or by other suitable means. Direct injection may also be used to load a cell or cells with a drug during efflux experiments.

For example, sensitive and drug-resistant cells may be loaded with doxorubicin by incubation of the cells in efflux medium containing about 5 μM doxorubicin with or without a blocking agent, such as 100 μM Verapamil™, for one and a half hours to ensure that both types of cell reach similar intracellular drug concentrations during incubation. (Verapamil™ is used for some experiments to block active efflux by P-gp in the CH<sup>R</sup>C5 cells. For comparison, the same concentration of Verapamil™ is preferably used in the AUXB1 cells as well).

The drug-loaded cells are preferably washed with 37°C drug-free efflux medium (e.g., NaCl 140 mM, KCl 5.4 mM, Hepes 5.5 mM, CaCl<sub>2</sub> 2.5 mM, MgCl<sub>2</sub> 0.5 mM and



glucose 11 mM adjusted to pH 7.4 with 1 M NaOH) with or without 100  $\mu$ M Verapamil™ 5 times to remove traces of the drug from the exterior of the cell(s).

The washed cells are quickly transferred to fresh efflux medium on the substrate 58 or other efflux container, and then placed on the stage of a Diaphot or confocal 5 fluorescence microscope. This transfer process preferably takes place as quickly as possible, so that the early stages of drug efflux can be monitored. By careful preparation, drug efflux measurements can begin within about 3 min after the last moment of incubation. Efflux is measured in the efflux medium with or without Verapamil™.

Control experiments may be carried out in the same way, but without any cells. for 10 example, a tissue culture dish containing MEM  $\alpha$  medium with 5  $\mu$ M doxorubicin and 100  $\mu$ M Verapamil™ may be incubated for one and a half hours and washed five times in the same way. The CFDE working electrode is then placed close (about 5  $\mu$ m) to the bottom of the dish and the detection carried out as for the live cells.

With reference now to FIGURE 8, a system for measuring drug efflux while 15 simultaneously measuring other properties, such as changes in pH, oxygen concentration, ion concentration, such as calcium and or potassium ion concentration, biomolecules, pharmacological agents, neurotransmitter sensing, metabolites of injected molecules, and the like of the efflux medium is shown. A carbon electrode 120, calcium sensor 122 and pH detector 124 define segments of an annular ring of radius  $r$  around a hydrophilic region 20 126 on a substrate 128 for simultaneous detection of these parameters. The electrode 120, calcium sensor 122 and pH detector 124 are connected with suitable electrochemical monitoring equipment 130, calcium detection equipment 132, and pH monitoring equipment 134, respectively. A retaining wall 136 retains the efflux medium 28 on the substrate. Alternatively, the efflux medium is retained by forming a well or other 25 depression in the substrate. The annular segmented arrangement allows for each of the detectors to be equally spaced from the cell, so that the properties of the medium should be the same at each detector.

Alternative arrangements are also contemplated, for example, the detectors 120, 122, 124 may be arranged in concentric rings around the hydrophilic area.

30 Several or many cells 14 or islets of small cell populations may also be surrounded each by a set of sensors inside the same medium or well. To study spatial distribution of chemicals, a matrix of dot like sensors (preferably, of multiple types) may be used. Such sensors may be used in conjunction with mapping drug efflux and its spreading around cell architectures, mapping of neurotransmitter release from nerve cells and cell networks, or

mapping of drug transport, pH, oxygen and the like inside tissues such as an excised tumor (the sensor array is preferably under the flat tissue side and around it on the substrate, optionally also monitoring chemicals around the tissue piece studied).

With reference to FIGURES 9 and 10, the drug or other chemical to be studied can  
5 be introduced directly into the cell, rather than by absorption through the cell membrane. A drug supply system, such as a diffusional microburette 140, is positioned below a hydrophilic cell attachment site 142 on a substrate 143 at which a cell 144 is attracted. The cell is allowed to settle on the site, and form a seal thereon (the base of the cell forms a sufficient seal on the site that chemicals do not tend to leak from the cell when the base  
10 of the cell is punctured). A capillary tube 146 which defines the tip of the diffusional microburette is positioned in a bore or aperture 147 in the substrate, preferably with an open end 148 flush with the substrate surface. The tip size is preferably in the micron range - from 10 microns or less. Tips and submicron outerdiameter can be formed. The tube is then pushed upwardly, through the aperture 147 in the substrate, in the direction  
15 of arrow F, to pierce the cell membrane, such that the open end 148 of the tube 146 is positioned inside the cell. To move the tube upwardly, a micromechanical motor 150 such as a piezoelectric actuator can be used. The tube preferably includes a diffusion membrane plug 152, formed from a medium such as an agar gel, polyacrylamide, or hydrogel, through which the chemical 154 diffuses into the cell from a source container 156,  
20 attached to a lower end of the tube 146. The diffusion membrane prevents convective flow through the tip, but enables diffusional transport (in either direction, dependent upon the concentration gradients inside the tip region), plus also electrical quantities can propagate through the tip. This same device can also be used to inject electrical quantities or measure electrical quantities, such as transmembrane voltages for the punctured cell. It  
25 can also be used to do both tasks (chemical delivery plus electrical input/detection) and, also, material subtraction from a cell.

Efflux of the chemical from the cell can be measured as before, using an electrode, such as a ring electrode 160, deposited on the substrate in an annulus around the cell, and connected with suitable electrochemical monitoring equipment 161 (see FIGURE 9). The  
30 electrode 164 measures concentrations of the chemical in a medium 162 surrounding the cell. Additionally, or alternatively, an electrode 164 can be positioned inside the cell itself, as shown in FIGURE 10. In the embodiment of FIGURE 10, the electrode 164 is positioned inside the tube of the diffusional microburette, and enters the cell when the end

- 24 -

148 of the tube pops up into the cell. The electrode is connected with suitable electrochemical monitoring equipment 166.

Such an arrangement can be used for studying efflux, influx, or influx and efflux simultaneously, by using both internal and external electrodes/sensors. In another embodiment, electrode 164 is a silver/silver chloride reference electrode and electrode 160 is a working electrode. This allows transmembrane voltages to be measured, for electrophysiological studies on a cell or cells.

With reference to FIGURES 11 and 12 a system suited to the study of cell monolayers or closely spaced cells is shown. For studying a monolayer of cells such as a 2-dimensional section of tissue 170, it is desirable to have medium 172 both above and below the cell (on the basolateral side). To achieve this, the monolayer of cells is positioned on a substrate mesh 174, which may be coated with a layer of protein 176, or other cell attracting material. For human cells, the spaces 178 between the mesh are of the order of 5-20 $\mu$ . A reservoir 180 holds medium 172 both above and below the mesh. The layer of cells 170 is allowed to settle and to grow on the mesh and electrochemical or other measurements made on the cells. For example, an electrode 182 can be positioned in the medium for efflux measurements. Additionally, or alternatively, electrode(s) 184 can be positioned on an upper surface of the mesh, or electrodes 188 may be positioned on the internal perimeter of the mesh hole. Suitable electrochemical monitoring equipment 190, 192 is connected with the electrodes. Additionally, electrodes 194, 196 may be positioned on the base, or elsewhere in the reservoir, a spaced distance from the cells, for bulk measurements on the medium and connected with electrochemical monitoring equipment 197.

For perfusion studies, two liquid ports 198, 199 are connected with the reservoir, one for liquid entry, the other for outflow.

To form such a system, a reservoir 180 may be formed from silicon, or other suitable material. The silicon reservoir is then filled, up to the desired position of the mesh with a chemical that can be later chemically etched out (removed) once the entire structure is complete. The chemical sets to form a support surface at the desired position of the mesh. A layer of material, for example, silicon is then laid down on the surface of the chemical, and patterned, for example with lithographic techniques, to define the mesh and spaces therebetween. Lithographic techniques can also be used for laying down the electrodes 184 and 188, and for laying down suitable wiring 200 (only two wires are shown for convenience) for connecting the electrodes to the monitoring equipment, for

example, on the underside of the mesh (or on top of the mesh, protected by an electrical insulation layer). Other sensors 202, such as pH or oxygen sensors, may also be formed on the mesh, with suitable connections to monitoring equipment (not shown). Other features, such as microburettes 204 (only one is shown) can optionally be positioned in  
5 the reservoir prior to or after addition of the chemical., e.g., while the chemical is setting.

Once all the layers have been laid down on the support surface provided by the set chemical, the chemical is etched away, for example, by dissolution in a suitable solvent, leaving the mesh and attached electrodes and other features in position. The reservoir can then be filled with an efflux medium 172, and the deposition of cells and testing carried  
10 out. There are thus essentially two reservoirs, one below the mesh and the other above it.

With reference now to FIGURE 13, a system for studying transport of a drug or other chemical across a gap junction 210 is shown. Gap junctions form between adjacent cells (e.g. in cell tissue) to allow communication between the cells. By using a mesh 174, as shown in FIGURES 11 and 12, or other suitable substrate, such as a layer of substrate  
15 with adjacent attractive sites on the surface the cells can be positioned for monitoring. Electrodes 212, 214 are positioned to be popped up into adjacent cells 216, 218 using micromotors (piezo actuators) 220, 222. Preferably, at least one diffusional microburette 224 is positioned to deliver a chemical, such as a drug, to one of the cells. FIGURE 13 shows two microburettes 224, 226, each one having its own electrode inside the pop-up  
20 tube. The electrodes are connected to suitable electrochemical monitoring equipment 228. By measuring voltages or currents between the two electrodes 212, 214 over time, the flow of the chemical across the gap junction(s) 210 between the two cells can be determined. In another embodiment, the gap junctions are studied by monitoring currents between adjacent cells arising from electrolyte movement between the cells without any addition  
25 of a chemical.

With reference now to FIGURE 14, a system suitable for making measurements on a tumor 240, or other three dimensional mass (tissue) of cells 242 is shown. A tumor or other mass of cells to be examined is sectioned to form a flat base 244. A substrate 246 is coated or otherwise formed with a cell-attracting site 248 of sufficient dimensions to  
30 adhere or attract the base of the tumor. Or, the cells are not attached to the substrate but are attached only to each other as in a tumor. This makes the sensors underneath less prone to fouling. On the site are formed an array of electrodes 250 or other sensors. The sensors may be spaced, for example to allow for study of variations in drug transport on the tissue level. Influx and/or efflux into/from the tumor as a whole, or other property,

across the radius  $r$  of the tumor, as shown in FIGURE 14, can be studied, mimicking treatment. Or, full 2 dimensional mapping can be obtained, and continued even outside the tumor. Thus, the attractive area does not have to be circularly symmetrical. Diffusional burettes, with or without integral electrodes, can be employed with such a system, as previously described. Using such a system the differences in efflux rates within the tumor can be studied. It has been found that efflux rates from cells in the center of a tumor differ from those outside, thus important information on drug dosage for tumors of a given size can be obtained.

By using electrochemical cleaning, adsorptive preconcentration, and the electrocatalytic effect due to ambient oxygen, 10 nM doxorubicin can be easily measured using even desensitized electrodes, and concentrations as low as 1 nM may be measured by electrodes that are not desensitized.

Desensitized means that the sensor loses over time a part of its response capability, thus decreasing the sensitivity towards the measurand. This is due to surface fouling by environmental factors, such as proteins present in the cell culture medium, efflux medium, or substances directly coming from the cell(s) studied.

The results can be compared with published data obtained on the same two cell lines in large populations with fluorescence or radiolabelled drug retention measurements. The initial intracellular drug concentrations for sensitive and resistant cells were similar in these experiments, too. (The incubation concentration for the resistant cells was 3 times higher than for the sensitive cells in one study, while an incubation with Verapamil<sup>TM</sup> was used in another. The ratio of the initial rates of efflux from the resistant versus sensitive cells found in this work (5.1) is consistent with published findings: about 4 in one study 32, and about 6 in another. (The anticancer drug used in this latter was daunorubicin whose structure is very similar to doxorubicin.) The time constants of the entire efflux process for both the resistant and sensitive cells obtained in this work are significantly shorter than those shown in prior published results:  $3.4 \pm 0.5$  min versus about 7 min for MDR cells, and for sensitive cells,  $10.2 \pm 2.9$  min versus about an hour that can be estimated from the drug retention curves in populations. This discrepancy may be due to two effects: (1) The background concentration of the efflux medium in the present experiments is virtually zero compared to a finite, and growing, level in effluxing cell populations. (2) Semispherical diffusion in the present experiments is also more efficient than the complicated diffusion patterns existing in suspended populations. Both effects lead to more efficient efflux in the single cell based scheme in the long term (apart from

the initial few minutes when spherical diffusion patterns from individual cells do not overlap in populations).

The importance of mass transport *outside* the effluxing cells is further stressed by the extreme discrepancy between this work and prior studies: the same cells in the form of a dense monolayer with similar methodology (CF microcylindrical electrode) produced a very long efflux process (constant or even increasing local concentrations during 1-2 hours). This can be explained by the sluggish dynamics of unidirectional diffusion (decreasing the ability of the medium to take up drug molecules from cells) compared to very efficient semispherical diffusion in this work. Suspended cell populations represent a combined behavior: efficient spherical diffusion initially which slows down after the individual diffusion patterns begin to overlap. This is illustrated by both the time constant data, the slopes, and overall shapes of the efflux curves obtained in prior studies.

Adsorption of the drug molecules onto the CFDE is significant only during the initial period of preconcentration but it becomes negligible at the end of the 1 min measurement period when the actual quantitation step takes place. Thus, local depletion by the electrode itself cannot cause the discrepancies between this work and prior published results: they must be independent of the sensing scheme employed.

Cell-to-cell micro-heterogeneity of the same type of cells could also be assessed because of the availability of efflux information from single cells. The drug efflux properties for AUXB1 cells do not vary too much: the local concentration at  $t = 3$  min was  $0.15 \pm 0.04 \mu\text{M}$  ( $n = 10$ ). Larger deviations were observed, however, for CH<sup>R</sup>C5 cells: the same value was  $0.77 \pm 0.44 \mu\text{M}$  ( $n = 10$ ). One possible explanation of this finding might be that in sensitive cells drug diffusion across the plasma membrane is the only efflux pathway, which is relatively stable. On the other hand, efflux pathways for resistant cells contain both drug diffusion and active transport mediated by P-gp, which can be affected by the availability of ATP, the level of overexpression and the distribution of P-gp, and other factors.

Statistical distribution of these variables from cell to cell can be obtained from such measurements. Similar statistical analysis has been carried out on the exocytosis of catecholamines from a single bovine adrenal medullary cell providing useful physiological information.

### Theoretical Considerations

For relatively short times, an effluxing single cell can be considered as a semispherical source with a constant efflux rate,  $E$  (mol/s). Due to the very low background concentration of doxorubicin in the efflux medium in the experiments reported here, we can assume that the initial background concentration right after washing the cells is zero. The time-dependent concentration profile outside a single cell can then be obtained for this diffusion problem by standard methods, yielding

$$C(d,t) = \frac{E}{\pi D (R+d)} \left[ \operatorname{erfc}\left(\frac{d}{2\sqrt{Dt}}\right) - \exp\left(\frac{Dt}{R^2} + \frac{d}{R}\right) \operatorname{erfc}\left(\frac{\sqrt{Dt}}{R} + \frac{d}{2\sqrt{Dt}}\right) \right] \quad (\text{A1})$$

where  $C$  = concentration

$d$  = the distance between the working electrode (CFDE) and the cell membrane

15  $t$  = time from beginning of efflux

$E$  = the efflux rate (mol/sec)

$\operatorname{erfc}$  = the error function, or complement of  $\operatorname{erf}$  ( $1 - \operatorname{erf}$ )

$D$  = the doxorubicin diffusion coefficient

$R$  = the cell radius

20 while the steady-state concentration profile will be

$$C_s(d) = E/2\pi D(R+d) \quad (\text{A2})$$

Since the electrode is positioned very close to the cell during drug efflux measurements,  $d \approx 0$ . Thus, the condition of reaching a steady-state recording is

$$[C_s(R) - C(R, t_s)]/C_s(R) \ll 1 \quad (\text{A3})$$

where  $t_s$  is time during steady-state electrode signal. Equations A1-A3 combined lead to

$$\exp(Dt_s/R^2) \operatorname{erfc}(\sqrt{Dt_s}/R) \ll 1 \quad (\text{A4})$$

which is equivalent to



$$\lim_{t \rightarrow \infty} \frac{\text{erfc}'(\sqrt{Dt}/R)}{\exp'(-(Dt/R^2))} = \frac{R}{\sqrt{\pi Dt_s}} \ll 1 \quad (\text{A5})$$

This requires  $t_s$  to be much larger than  $R^2/\pi D$  for ensuring steady state at the CFDE.

In experiments described herein,  $D=1.5 \times 10^{-6} \text{ cm}^2/\text{s}$ . Since the radius of suspended, and thus round, cells is  $\sim 8 \mu\text{m}$ , after the cells stretch out due to attachment to the culture dish or other substrate, the height of a cell is less than about  $5 \mu\text{m}$ . Since  $R^2/\pi D \approx 200 \text{ ms}$  even for  $R \approx 10 \mu\text{m}$ , it is safe to assume a quasi-steady concentration distribution around the studied cell to analyze our efflux data, which are obtained at a rate of 1 data point/min from processes whose overall time constant is in the range of minutes. Thus, diffusion patterns around the effluxing cell will adjust quickly to changes in efflux dynamics.

Since the radii of resistant and sensitive cells are similar, the distance between the CFDE and the cell center is also similar.

On the basis of Equation 2, the local concentration of doxorubicin detected by the CFDC is proportional to the total efflux rate,  $E$ , in a quasi-steady state. However, since the shape of attached cell has been found to be far from semispherical, a direct calculation of efflux rate from local concentrations is problematic. Positioning the CFDE further away from the studied cell can make the concentration distribution more semispherical, and thus, Equation 2 may become directly applicable for determining absolute efflux rates.

## 20 Data analysis

"Detection limits" are defined herein as the concentration corresponding to a signal that is three times the root mean square (rms) of background noise. To compare quantitatively the signal-to-noise (S/N) ratios for the fluorescence and electrochemical techniques, the "signal" is defined herein as the entire dynamic range of the efflux data from the resistant cells. After fitting an exponential curve to the data from the sensitive cells, the noise is defined as the rms of residual errors at the relatively stable portion of the efflux curve (from 10 to 15 min where the noise is  $0.011 \pm 0.004 \mu\text{M}$ ,  $n=10$ ).

To obtain the time constant of the entire efflux process from the single cell results as well as from cell population based results from the prior art, an exponential decay is assumed for both the rate of efflux (present data) and data on intracellular retention from the prior art. This is because the accepted definition of a time constant is tied to an exponential relationship, regardless whether the observed decay is truly exponential or of some other type. For prior art data, the time constant was thus determined as the efflux

time when drug retention is about 37% of its original value. The present data were evaluated by an exponential fitting.

### Single Cell Studies

5 In order to detect drug efflux from a single cancer cell rather than a number of cancer cells, with the above-described technique, it is preferable to use a 20- $\mu$ m-long cylindrical electrode, a microdisk CF electrode, or a ring electrode placed close to the effluxing single cell. Although signals from a single cell are generally small (the average signal increase per 6 minute measurement period detected with a 5-mm-long fiber is about  
10 10 nA/50 mV, thus the signal change due to efflux from an average single cell should be about 50 pA/50mV for every 6 minutes), signals of this magnitude are still accessible by the present method using microvoltammetry.

Results obtained with a CF microdisk electrode have shown that efflux from both single AUXB1 and CH<sup>R</sup>C5 cells can be monitored with the technique described. This is  
15 important because heterogeneities in efflux characteristics within one cell type (sensitive or drug-resistant line) cannot be characterized without a single-cell efflux measurement scheme.

The technique is also ideally suited to obtaining efflux data of high temporal resolution from the beginning of efflux, which renders determination of the parameters of  
20 both passive and active efflux numerically better conditioned than when only techniques of low temporal resolution are available.

It is known that a variety of other drugs, such as daunorubicin, marcellomycin, chlorambucil, fluorouracil, and methotrexate are also electroactive. Therefore, that efflux of these drugs from populations of cancer cells as well as single cancer cells may also be  
25 studied with the method described herein.

Voltammetry of doxorubicin and other electroactive drugs at a CF microelectrode, as described herein, is simple to perform and has excellent analytical characteristics for cell culture studies.

With an electrocatalytic effect due to ambient oxygen in the solution and  
30 adsorptive preconcentration of doxorubicin on the CFDE surface, significant signal enhancement is achieved, as compared to usual diffusion current based schemes. This enables the detection of doxorubicin concentrations down to 1 nM concentration. With a repetition rate up to 1 data point/30 s (10 s cleaning and 20 s preconcentration), the

technique introduced in this work can provide information with a temporal resolution much better than for conventional methods using cancer cell populations.

For assessing drug transport on the single cell level, a CFDE with microvoltammetry can provide a far better detection limit, sensitivity, and results than  
5 fluorescence detection. The fluorescence data are much noisier and less sensitive, even when the loading doxorubicin concentration is 4 times higher than in the present electrochemical method. In addition, the fluorescence based scheme can only provide indirect information on efflux, since the directly measured property is drug retention inside the cell. A method using a CFDE with microvoltammetry directly measures efflux, and  
10 simultaneously assesses also the actual driving force of efflux, since local, not average bulk, concentrations are what the P-gp transporters, as well as the CFDE, sense.

This enables information to be obtained on an unresolved area of MDR studies: whether a model involving a partitioning equilibrium and an indirect role of P-gp in MDR or an active pump model is correct. A comparison between initial results and published  
15 data (as outlined above) seems to favor the first hypothesis.

Information on micro-heterogeneity in terms of drug efflux dynamics between individual cells may also be assessed with these techniques. The results show a high non-uniformity from cell-to-cell in either the level of overexpression of P-gp, and/or in the distribution of P-gp in the plasma membrane of individual cells.

20 Information on initial drug efflux kinetics is valuable for testing mechanisms of P-gp related efflux, but this is not readily achieved with conventional techniques. Combining the approach presented here with a perfusion system provides a method of study. Optionally, simultaneous drug loading of a single cell by a Diffusional Microburette with CFDE detection enables monitoring of drug influx and efflux  
25 simultaneously at single cancer cells, thus assessing a complete mass balance of the studied drug for individual cells as a function of time. Thus, single cell based microvoltammetry can provide more relevant data for quantitative understanding and mathematical modeling of multidrug resistance, and more profound information about efflux kinetics than techniques developed and used thus far.

30 The techniques described have a wide variety of applications, both in analytical studies and in clinical applications. The techniques described allow a wide variety of cell architectures to be studied, from the simplest, a single cell, to monolayers of cells, and sections of tumors. In between these, it is possible to study the interactions of two or more cells by selecting the spacing between cells. This gives information, for example, about

how tumor cells interact with one another. Experiments can be carried out at increasing (or decreasing) cell spacings, to determine how these interactions are affected by cell spacing. Monolayers of cells can be studied, including epithelial and endothelial cells. The cells may be spaced or confluent (touching). Polarized monolayers, comprising a two-dimensional section of tissue may also be studied. These layers comprise a variety of different types of cells which have particular interactions one with another.

In the area of drug testing, the techniques described allow for rapid screening of new drugs, and the uptake/efflux by cells/cell populations. The techniques for cell positioning on a substrate using hydrophilic regions, the formation of electrodes by photolithographic techniques, and or drug delivery by microburette allow cells to be positioned on a substrate and large numbers of studies conducted rapidly, and without complex positioning equipment, microscopes, and the like.

A wide variety of cell disfunctions can be studied, including cancer growth, and other disfunctions, such as cystic fibrosis.

Without intending to limit the scope of the invention, the following examples demonstrate methods of measurement and the results which may be obtained with this method. The substrate/electrode arrays described can be produced inexpensively and in bulk, allowing for single use, or reuse if desired. By using arrays of test cells, statistical data can be obtained.

The system can be used in clinical studies, for example, for optimizing chemotherapy treatment for a particular patient. A biopsy is performed on the patient to obtain cancer or other cells to be treated. One or more of the cells are then deposited on a substrate, as described and one or more different drugs studied. From drug efflux measurements, or other electrochemical or chemical measurements on the cells, the optimum dosage and/or type of drug for treating the patient's condition can be determined.

A wide variety of physiological processes can be studied with the techniques described, including, but not limited to the transport of chemicals, including drugs, ions, pharmacological agents, and the like. Ionic balance, mass transport, and other aspects of cells can be studied.

### **Examples**

#### **Study of Doxorubicin Efflux from Cancer Cells**

Concentration changes near a single cell or a monolayer of cells induced by efflux of doxorubicin from the cell(s) have been monitored in this work by a CDCF electrode,

using adsorptive preconcentration followed by electrochemical measurements. The results were compared with measurements by fluorescence.

### Equipment and Materials

5       The cell lines used for the investigations were an auxotrophic mutant of Chinese hamster ovary cells, AUXB1, and its multidrug resistant (MDR) subline, CH<sup>R</sup>C5. These were obtained from Dr. V. Ling (Ontario Cancer Institute, Toronto, Ontario, Canada). Carbon fiber for the microelectrode core with a nominal diameter of 7.5  $\mu\text{m}$  was purchased from Zoltek (St. Louis, MO). Both polyethylene (PE) tubing (i.d. 0.38 mm, o.d. 1.09 mm) and glass capillary (i.d. 1.10 mm, o.d. 1.50 mm) were from Clay Adams (Parsippany, NJ).  
10       Teflon-coated Ag wire (World Precision Instruments) and a 18-gauge 1½-inch stainless steel hypodermic needle (Becton-Dickinson) were used to prepare the reference electrodes.

Crystalline doxorubicin (doxorubicin hydrochloride, Adria lot no. 89E07A; FIGURE 5) was obtained from Adria Laboratories (Columbus, OH). The efflux medium  
15       contained minimum essential  $\alpha$  medium (MEM  $\alpha$  medium), 25 mM HEPES, and 10% fetal bovine serum, with a pH adjusted to 7.2-7.4. Powdered MEM  $\alpha$  Medium was obtained from Life Technologies (Gaithersburg, MD). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT).

AUXB1 is the parental cell line that expresses little P-glycoprotein, while the  
20       derived CH<sup>R</sup>C5 cells express high levels of P-glycoprotein. Both cell lines were cultured in a tissue culture dish at 37°C in bicarbonate-buffered MEM  $\alpha$  containing 10% FBS.

The electrochemical system consisted of a CFDE working electrode (diameter  $\sim 7.5$   $\mu\text{m}$ , exposed length 5 mm) and a Ag/AgCl reference electrode (diameter 0.3 mm, length 1 cm) both in contact with the efflux medium ( $\sim 5$  ml) in the efflux container as shown in  
25       FIGURE 1. For electrochemical experiments, a CHI660 potentiostat was used with a preamplifier (CH Instruments, Cordova, TN). Control experiments not involving cells were performed inside the metal Faraday cage of this instrument. Cell experiments were done on top of an antivibration platform, on the stage of an inverted microscope (Diaphot, Nikon, Tokyo, Japan). The whole assembly was placed inside a temperature-controlled  
30       Faraday cage ( $\sim 37^\circ\text{C}$ ).

The data were transferred after each measurement via an I/O interface to a computer for storage. This computer was also used for controlling the BAS 100A to execute preprogrammed experiments.

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For fluorescence detection, a confocal microscope system (LSM 410 inverted Laser Scan microscope, Carl Zeiss, Oberkochen, Germany) was used with an excitation wavelength of 488 nm and a 40x objective. Emission above 590 nm using a long-pass optical filter was monitored.

5 A 4-5 cm fiber was aspirated into a 2-3 cm long PE tubing by a vacuum pump (Devilbiss, Somerset, PA), leaving about 1 cm CF extending from both ends. The tubing was then heated above a hot soldering iron and pulled to form a thin section in the middle. After touching the soldering iron very briefly to this section, only a submicron thick PE insulation remained around the CF. The assembly was then cut in the middle with a  
10 scalpel. The PE at the other end was attached to a glass capillary and connected via mercury to a conducting wire to make a CFDE (FIGURE 1). An Ag|AgCl wire was used as the reference.

#### Preparation of Cells for Cell efflux experiments

15 Before electrochemical or fluorescence detection, the cells were loaded with doxorubicin by incubation with 5  $\mu$ M doxorubicin with or without 100  $\mu$ M Verapamil<sup>TM</sup> for one and a half hours to ensure that both types of cell reach similar intracellular drug concentrations during incubation. Verapamil<sup>TM</sup> was used for some experiments to block active efflux by P-gp in the CH<sup>R</sup>C5 cells. For comparison, the same concentration of  
20 Verapamil<sup>TM</sup> was used in the AUXB1 cells as well.

The loaded cells were washed with 37°C drug-free efflux medium (NaCl 140 mM, KCl 5.4 mM, Hepes 5.5 mM, CaCl<sub>2</sub> 2.5 mM, MgCl<sub>2</sub> 0.5 mM and glucose 11 mM adjusted to pH 7.4 with 1 M NaOH) with or without 100  $\mu$ M Verapamil<sup>TM</sup> 5 times, and then placed on the stage of a Diaphot or confocal fluorescence microscope. Drug efflux measurements  
25 began 3 min after the last moment of incubation. Efflux was measured in the efflux medium with or without Verapamil<sup>TM</sup>.

Control experiments were carried out in the same way, but without any cells, i.e., a tissue culture dish containing MEM  $\alpha$  medium with 5  $\mu$ M doxorubicin and 100  $\mu$ M Verapamil<sup>TM</sup> was incubated for one and a half hours and washed five times in the same  
30 way. The CFDE was placed close (about 5  $\mu$ m) to the bottom of the dish and the detection started.

### Stabilization of the CFDE

Each CFDE was stabilized, prior to an efflux experiment, by running cyclic voltammetry (CV) from -200 to +600 mV at 100 mV/s twenty times in fresh efflux medium, and then Osteryoung Square Wave Voltammetry (OSWV) from -300 mV to -800 mV at 60 mV/s five times. (Doxorubicin detection using its cathodic peak was later done with OSWV and the above parameters. For anodic detection, OSWV scans from 200 to 600 mV at 60 mV/s were used.)

A -1000 mV potential was applied for testing electrochemical cleaning of CFDEs for 2, 5, 10 and 20 s to electrodes previously exposed to efflux medium containing 0.1  $\mu$ M or 1  $\mu$ M doxorubicin for 1 min. A cathodic OSWV scan was then applied to detect doxorubicin reduction current after each cleaning.

### Study of the Stability of Electrodes

To test the stability of the pre-stabilized CFDEs during measurements using the reduction peak of doxorubicin, the electrode was immersed into the efflux medium containing doxorubicin of fixed concentrations (0.2, 0.5 and 1  $\mu$ M) which lie within the range typical of single cell efflux experiments (0.1 to 1.5  $\mu$ M). Cathodic OSWV runs were performed every 1 min, after a 10 s cleaning and 20 s preconcentration (open circuit) step.

### Regeneration of Working Electrode Surface Between Measurements

Experiments were conducted to determine appropriate times for regeneration of the working electrode between data points. The shortest cleaning pulse applied (2 s) was not generally sufficient to regenerate the surface. A 5 s pulse almost completely cleaned a CFDE after contact with a buffer containing 0.1  $\mu$ M doxorubicin. A 10 s pulse cleaned the electrode even after contact with 1  $\mu$ M doxorubicin (only about 4% of the original signal remained). A 20 s cleaning did not significantly improve cleaning efficacy. Therefore, since the 0.1 and 1.5  $\mu$ M solutions bracket the typical concentration range encountered close to single cells in the present efflux experiments, a 10 s cleaning cycle was adopted.



### Preconcentration Studies

Drug adsorption can be used for preconcentration of doxorubicin on the CFDE surface.

5        The dynamics of drug adsorption onto the stabilized CFDE surface were studied in oxygen-free efflux medium (purged with nitrogen for half an hour) containing 0.1 or 1  $\mu\text{M}$  doxorubicin. OSWV from -300 to -800 mV at 800 mV/s was then applied to detect the cathodic peak at intervals of 4 s immediately after a freshly stabilized CFDE was immersed into the solution. The results were recorded.

10       The response of stabilized electrodes to changing doxorubicin concentrations were tested with cathodic OSWV (60 mV/s) after the same cleaning and preconcentration steps as above, in the range of 0.1 to 1.5  $\mu\text{M}$  in efflux medium, first increasing and then decreasing the concentration.

      With reference to FIGURE 15, cathodic OSWV peaks revealed an exponential  
15 surface saturation process. FIGURE 15 is a plot showing the dynamics of doxorubicin adsorption onto a stabilized CFDE surface in oxygen-free efflux medium containing 1  $\mu\text{M}$  doxorubicin. OSWV from -300 to -800 mV at 800 mV/s was applied to detect the cathodic peak after a freshly stabilized CFDE was immersed into the solution. FIGURE 16 shows individual OSWV sweeps obtained at every 4 s. The diamonds represent the heights of  
20 the differential current peak determined from the OSWV curves. The nominal time of electrode immersion is at  $t=0$ . Solid line: exponential saturation curve least-squares fitted to the data; A time constant of  $9 \pm 3$  s, was obtained which is almost independent of the drug concentration within the 0.1 – 1.5  $\mu\text{M}$  range. (Since at 800 mV/s one scan took only about 0.6 s, this did not influence much the seconds precision obtained. The slight delays  
25 observed, such as about 0.2 s in FIGURE 15, are due to the finite speed of mechanical handling and immersion of the electrode, and also to the time needed for the scan to reach the reduction range of doxorubicin.)

      Based on these results, a 20 s adsorptive preconcentration period was adopted that corresponds to about 90% completion of the adsorption process. Also, small errors in this  
30 period will not affect significantly the results, due to the relatively small slope of the dynamic saturation curve at  $t = 20$  s.

      Since adsorption currents are proportional to scan rate, this can also be used to amplify the signals. Relatively high scan rates (800 mV/s) are preferred to provide high temporal resolution for adsorption kinetics studies. The higher scan rate, in turn,

rationalizes the negative shift of the peaks (see FIGURE 16) with respect to the -650 mV usually observed.

### Single Cell Studies

5 To monitor efflux from a single cancer cell, the tip of a stabilized CFDE was moved to touch a suitable cell slightly and then withdrawn to about 1  $\mu\text{m}$  away from the cell. A fine three-axis hydraulic micromanipulator (MO-203, Narishige, Tokyo, Japan) mounted on a Diaphot microscope was used for these manipulations. Local drug concentrations were measured using the same cathodic OSWV procedure as defined above  
10 (60 mV/s). One measurement per minute (after a 10 s cleaning and a 20 s preconcentration step) was performed to obtain the time course of efflux during 15 min. In some experiments, the anodic protocol was also used to test consistency of the results independent of the electrochemistry involved. The calibration of the CFDE was carried out immediately after each drug efflux experiment in the 0.1 to 1.5  $\mu\text{M}$  range. The  
15 temperature of the efflux medium during calibration was 37°C, and the measurement protocol was the same as for the efflux measurements.

Doxorubicin efflux from AUXB1 and CH<sup>R</sup>C5 single cells was monitored with or without 100  $\mu\text{M}$  Verapamil<sup>TM</sup> in the efflux medium. (FIGURES 17-18 show the results in the absence of Verapamil<sup>TM</sup>).

20 FIGURES 19 and 20 are plots showing the results of drug efflux measurements from a single sensitive (AUXB1) and a drug-resistant (CH<sup>R</sup>C5) cell in efflux medium. A stabilized CFDE was used to obtain the time course of efflux during 13 min with one measurement per minute. Each measurement consisted of a 10-second cleaning of the CFDE at -1000 mV and a 20-second preconcentration, followed by doxorubicin detection  
25 using OSWV with a scan from -300 to -800 mV. The data from the first, second, third, and following odd-numbered sweeps from the experiment are shown in FIGURE 21, which shows local concentrations of doxorubicin vs efflux time for an AUXB1 and a CH<sup>R</sup>C5 cell. The measurements were carried out in the absence of Verapamil<sup>TM</sup>.

30 Peak currents of OSWV,  $\Delta i_p/\Delta E$ , were determined graphically from the curves (such as those shown in FIGURES 19 and 20) and then local concentrations were back-calculated based on postexperimental calibration. FIGURE 22 shows a calibration of a CFDE for doxorubicin, plotting the peak height,  $\Delta i_p/\Delta E$ , of the cathodic OSWV peaks at -650 mV versus Ag|AgCl in efflux medium against a logarithmic concentration axis. The concentration range covers typical drug concentrations encountered at effluxing single

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cells. The inset shows the individual OSWV sweeps whose peak heights were used to construct the calibration curve.

The cells were loaded with doxorubicin, as previously described. At time  $t=0$ , washing of the cells began. The first measurement began at  $t=3$  minutes, due to the finite time needed for multiple washings and CFDE positioning. Clear and well shaped OSWV peaks were observed around -650 mV (see FIGURES 17 and 18).

### Fluorescence experiments

Fluorescence images were sampled by a CCD camera (a digital image gathering camera consisting of a matrix of tiny individual photodetectors, typically solid state devices, for acquiring digital images) at intervals of 1 min. To compare fluorescence intensities from both cell lines, all parameters for the microscope, once optimized, were kept fixed. Doxorubicin molecules can bind to cell nuclei. For the sensitive cell line, local fluorescence intensities in the nucleus can be about 3-5 times higher than in the cytoplasm of the same cell. The nuclei of sensitive cells can also bind more drug molecules than resistant cells. On the other hand, a part of doxorubicin fluorescence can be quenched by DNA molecules in the nuclei. Also, efflux depends on the concentration of the free (unbound) drug in the cytoplasm. Therefore, average fluorescence intensities were measured in the cytoplasm of cells excluding the nucleus. Since relative measurements were performed, no calibration was needed.

### Study of the Effect of Oxygen on Voltammetric Detection of Drug Efflux

To establish the effect of oxygen on voltammetric detection (electrocatalytic effect), three beakers containing the efflux medium were bubbled with nitrogen, air, or pure oxygen for half an hour. Doxorubicin was then added to a final concentration of 0.1  $\mu\text{M}$ . A stabilized and electrochemically cleaned CFDE was used to detect in each beaker both the anodic and cathodic peak with the respective OSWV scans. Several CFDEs were used to repeat these measurements to characterize statistically the results.

FIGURE 17 is a plot showing the catalytic effect of oxygen on doxorubicin detection. Doxorubicin solutions with 0, 21, and 100% oxygen concentrations were prepared by bubbling the solutions with  $\text{N}_2$ , air, or pure oxygen, respectively, for 0.5 h. OSWVs from -800 to -300 mV or from 200 to 600 mV were applied to the same stabilized CFDE to detect the cathodic and the anodic peaks in 0.1  $\mu\text{M}$  doxorubicin solution. After

post-experimental calibration, the local concentration of doxorubicin directly adjacent to the cell membrane versus efflux time was obtained.

Much higher currents are observed when cathodic OSWV is used, as compared to the anodic scheme under otherwise identical circumstances, even in a medium without  
5 oxygen (the ratio of cathodic vs. anodic peak height is about 5 to 1). This may be due to the fact that doxorubicin is positively charged at physiological pH, which may loosen the adsorptive binding between the CF surface and the adsorbed drug molecules during the anodic scan.

The cathodic peak is further enhanced in a medium equilibrated with ambient air  
10 (21% oxygen), as compared to that obtained in deoxygenated solution, under otherwise identical conditions (FIGURE 17). The same CFDE was used to detect both doxorubicin peaks at different oxygen concentrations. No positive effect of oxygen on the anodic peak was found. In fact, the peak even decreases significantly at 100% oxygen, likely caused by a competitive chemical oxidation of bulk doxorubicin. On the other hand, 21% oxygen  
15 increases the cathodic peak instantaneously about 5-6, times compared with deoxygenated solution, and an amplification factor of 20-25 was found in 100% oxygen. OSWV curves in doxorubicin-free solutions containing 21% and 100% oxygen (not shown) exhibited no peak in the potential range from -300 to -800 mV. This showed that it was indeed the catalytic effect of oxygen that enhanced the cathodic peak in actual efflux measurements.

20

### Determination of the Effective Surface Area of the Working Electrode

Chronoamperometry with a potential step from 0 to -500 mV for a duration of 10 s in 3.0 mM ruthenium hexamine trichloride + 0.1 M KCl solution was performed to  
25 determine the effective surface area of the fabricated CFDEs. A value of  $r$  larger than 4.5  $\mu\text{m}$  may mean that the PE insulation is not intact and therefore, such electrodes were discarded. The average calculated radius of the electrodes used was  $3.81 \pm 0.66 \mu\text{m}$  ( $n = 10$  and  $\alpha = 4$ ), which is almost equal to the expected nominal value ( $d = 7.5 \mu\text{m}$  for the CF used). This is because, unlike beveled CFDEs, the electrodes in this work were obtained  
30 by an almost perpendicular cut of the fiber.

**CFDE stability, sensitivity, and limit of detection for doxorubicin**

Five stabilized CFDEs were used to measure doxorubicin in the efflux solution containing fixed concentrations of the drug (0.1 - 1  $\mu$ M), equilibrated with air. The average decrease in cathodic OSWV peak height during 20 min in the trials was about 8% (1 measurement/min). A typical efflux experiment lasted about 15 min. In this time, sufficient stability was achieved by the stabilization protocol described above.

Electrode response to drug concentration changes was also studied. For reasons mentioned before, without a cleaning procedure the electrode response did not change instantaneously when the doxorubicin concentration decreased. However, 10 s electrochemical cleaning can sufficiently clean and regenerate the electrode surface. With such cleaning and 20 s preconcentration, the height of the cathodic OSWV peaks versus doxorubicin concentrations produced a log-linear calibration graph (not shown; peak height in pA/25mV =  $156 \log(C \text{ in } \mu\text{M}) + 168$ ;  $r^2 = 0.993$ ). The plausible explanation of the shape of the calibration curve is that in the studied 0.1 - 1.5  $\mu$ M concentration range adsorption current is dominant, and adsorption isotherms are often log-linear. To test reproducibility, electrodes were calibrated first from high to low, and then from low to high concentrations. The average peak height difference between these two calibration curves is only 10 pA/25 mV in the concentration range of 0.1 - 2  $\mu$ M, which is only 5% of the dynamic range. This showed that a properly stabilized and cleaned CFDE can respond to doxorubicin concentration changes with a reproducibility reasonable for a microelectrode to be used in a live biological preparation.

Although the preconditioning protocol was sufficient to make the electrode almost completely stable (comparing pre- and post-experimental calibration, the average desensitization observed during an efflux experiment was 13%). Therefore, post-experimental calibration was used to interpret drug efflux results. The reason for this choice is that desensitization of a CFDE by proteins and other biochemicals usually happens in the first moments when the electrode approaches a live cell, and the electrode will become quite stable afterwards.

By using electrochemical cleaning, adsorptive preconcentration, and the electrocatalytic effect due to ambient oxygen, 10 nM doxorubicin can be easily measured

using even desensitized electrodes, and concentrations as low as 1 nM could be measured by electrodes that were not desensitized.

#### Electrochemical and fluorescence control experiments

5 To test the effectiveness of the washing procedure before efflux, doxorubicin was measured after a standard washing procedure about 5  $\mu$ M away from the bottom of the dish. The results showed a residual signal corresponding to about 0.02  $\mu$ M. Although the time course of this signal was also recorded, unlike in drug efflux experiments, this signal was found to be quite constant. Therefore, this background signal was subtracted from all  
10 efflux results.

Because the only major difference between the AUXB1 and CH<sup>R</sup>C5 cell lines is overexpression of P-gp in the plasma membrane of the CH<sup>R</sup>C5 cells, and 100  $\mu$ M Verapamil<sup>TM</sup> can almost completely block P-gp, both cell lines should have the same intracellular doxorubicin concentration after being incubated in 5  $\mu$ M doxorubicin and 100  
15  $\mu$ M Verapamil<sup>TM</sup> for one and a half hours. Fluorescence microscopy was used to test this hypothesis, since fluorescence intensity is in general proportional to doxorubicin concentration. By detecting intensities of loaded sensitive and resistant cells of similar size and thus, with similar optical pathlengths (while excluding the nuclei), the same fluorescence intensities (within experimental error) were obtained for the cytoplasm of  
20 both cell lines: after background subtraction, the relative fluorescence intensities were  $57.0 \pm 4.8$ , and  $56.3 \pm 4.9$  ( $n = 20$ ) for the sensitive and resistant cells, respectively. This showed that efflux began from the same starting intracellular concentration in both cell types.

To corroborate the electrochemical results, fluorescence was also used to obtain  
25 the time course of drug retention. The results (not shown) indicated that, although the patterns of drug efflux for the sensitive as well as MDR cells were reasonable, in the encountered intracellular drug concentration range the data are very noisy. The S/N ratios for fluorescence versus the electrochemical technique are about 15 and 60, respectively. Considering also that four times higher doxorubicin concentration was used for incubation  
30 in the fluorescence efflux experiments and thus, much higher intracellular doxorubicin concentrations were to be monitored by fluorescence than the extracellular concentrations determined by a CFDE, both the sensitivity and the detection limit of the electrochemical

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technique are much better. (Both intracellular fluorescence microscopy and the extracellular electrochemical technique assess, albeit indirectly, the same variable: drug efflux rate, and hence, the comparison is meaningful.)

Because an effluxing single cell attached to the bottom of a culture dish can be  
5 perceived as a microscopic semispherical source, diffusion of doxorubicin away from the studied cell is at quasi-steady state over the 15 min time scale of an experiment. This makes it possible to correlate the measured concentrations with actual efflux values. Decreasing peaks mean decreasing efflux over time for both cell lines.

The kinetics of drug efflux from CH<sup>R</sup>C5 cells into an efflux medium containing  
10 Verapamil<sup>TM</sup> are similar to those of AUXB1 cells (not shown, similar to AUXB1 data in Figure 21). This is because 100  $\mu$ M Verapamil<sup>TM</sup> can almost entirely block P-gps in CH<sup>R</sup>C5 cells, thus making the efflux from resistant cells purely due to passive diffusion only, which is the only pathway for drug efflux from sensitive cells.

Without Verapamil<sup>TM</sup> present during efflux, the drug resistant CH<sup>R</sup>C5 cells exhibit  
15 a much higher initial efflux rate and shorter time constant of the entire process than AUXB1 cells (Figure 21): the efflux rate at  $t = 3$  min for CH<sup>R</sup>C5 cells is about 5.1 times higher than for AUXB1 ( $n = 10$ ). This means that about 4.1 times more efflux is produced by active transport mediated by overexpressed P-gp in the resistant cells than by passive efflux at the same intracellular concentration.

20 The invention has been described with reference to the preferred embodiment. Obviously, modifications and alterations will occur to others upon reading and understanding the preceding detailed description. It is intended that the invention be construed as including all such modifications and alterations insofar as they come within the scope of the appended claims or the equivalents thereof.



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Having thus described the preferred embodiment, the invention is now claimed to be:

1. A method of measuring efflux of a chemical (26) from a cell (14, 5 52, 144, 216, 218), or a population of cells (170, 240, 242) the method including introducing the chemical to the cell and measuring an electrochemical property of a medium (28, 152, 162, 172) surrounding the cell with an electrochemical system (A) which includes a working electrode (20, 50, 120, 160, 164, 180, 184, 188, 212, 214, 250) and a reference electrode (24, 54), the property being related to a concentration of the 10 chemical in the medium, the method characterized by:

adding oxygen to the medium to increase a signal strength of the electrochemical property.

2. The method of claim 1, further characterized by:  
15 preconcentrating the chemical in the medium on the working electrode prior to the step of monitoring the electrochemical property of the medium.

3. The method of either one of preceding claims 1 and 2, further characterized by:  
20 the cell population comprising a monolayer of cells.

4. The method of either one of preceding claims 1 and 2, further characterized by:  
the cell being a single cell.  
25

5. The method of any one of preceding claims 1-4, further characterized by:  
the step of adding oxygen to the medium including bubbling an oxygen-containing gas into the medium.  
30

6. The method of claim 5, further characterized by:

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the step of bubbling an oxygen-containing gas into the medium including bubbling a gas having a greater oxygen concentration than ambient air into the medium.

7. The method of any one of preceding claims 1-6, further  
5 characterized by:

prior to the step of measuring an electrochemical property of the medium, positioning the cell or population of cells on a substrate (70, 84, 128, 143, 174, 246), a surface (74) of the substrate having at least one hydrophilic region (72, 72', 126, 142) to which at the cell or the population of cells attaches, the hydrophilic region being  
10 surrounded by a hydrophobic region which resists attachment of cells to the surface.

8. The method of claim 7, further characterized by:  
the hydrophilic region being sized to permit attachment of only one cell.

15 9. The method of either one of preceding claims 7 and 8, further characterized by:

the substrate (70) including a number of spatially orientated hydrophilic regions (72', 96) for spatially orienting a preselected number of cells or groups of cells.

20 10. The method of any one of preceding claims 7-9, further characterized by:

the working electrode being a carbon ring electrode (50) , formed on the surface (74) of the substrate (70) .

25 11. The method of any one of preceding claims 7-10, further characterized by:

the step of introducing the chemical to the cell including injecting the chemical into the cell.

30 12. The method of claim 11, further characterized by:

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the step of injecting the chemical into the cell including forming an aperture (147) in the substrate and allowing the chemical to diffuse into the cell from an injection tube (146) of a diffusional microburette (140, 204, 224, 226).

5                   13.    The method of any one of preceding claims 1-12, further characterized by:

the chemical being a drug used for the treatment of cancer.

10                   14.    The method of any one of preceding claims 1-13, further characterized by:

the step of measuring an electrochemical property including voltammetric scanning in the region of a cathodic peak of the chemical.

15                   15.    The method of claim 1, wherein the step of measuring the electrochemical property includes:

(a) preconcentrating effluxed chemical on the working electrode;

(b) detecting a function of the current flowing between the working electrode and a second electrode;

20                   (c) cleaning the electrode to remove traces of the chemical from the working electrode;

(d) comparing the function obtained in step (b) with a calibration curve to obtain a measure of a concentration of the chemical in the medium; and

repeating steps (a) - (d) to obtain a plot of concentration over time.

25                   16.    An apparatus for measuring efflux of a chemical from a biological cell (14, 52, 144, 216, 218), or a population of cells (170, 240, 242), the apparatus including:

a substrate (70, 84, 128, 143, 174, 246) having a surface (74) which receives the cell;

a medium (28, 152, 162, 172) on the substrate;

30                   an electrochemical monitoring system (A) which measures an electrochemical property of the medium surrounding the cell, the property being related to a concentration of the chemical in the medium, the apparatus characterized by:

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the substrate surface having at least one attachment region (72, 72', 126, 142) to which the cell or population of cells attaches, the region being surrounded by a resistant region (78) which resists the attachment of cells.

5                   17.     The apparatus of claim 16, further characterized by:  
a source (156) of an oxygen containing gas which supplies oxygen to the medium for increasing the signal strength of the electrochemical property.

10                   18.     The apparatus of claim 17, further characterized by:  
the source of the oxygen containing gas comprising a container of substantially pure oxygen under pressure.

15                   19.     The apparatus of any one of preceding claims 16-18, further characterized by:  
the electrochemical system including a carbon electrode (20, 50, 120, 160, 164, 180, 184, 188, 212, 214, 250) which is positioned adjacent the at least one attachment region for measuring the electrochemical property of the medium surrounding the cell.

20                   20.     The apparatus of claim 19, further characterized by the carbon electrode (50, 160, 164, 180, 184, 188, 212, 214, 250) defining an annulus which surrounds the at least one attachment region.

25                   21.     The apparatus of any one of preceding claims 16-20, further characterized by:  
the hydrophilic region being sized for attachment of only one cell.

30                   22.     The apparatus of any one of preceding claims 16-21, further characterized by:  
the substrate including a number of spatially orientated hydrophilic regions (72') for spatially orienting a preselected number of cells or groups of cells.

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23. The apparatus of any one of preceding claims 16-22, further characterized by:

a sensor (122, 124) formed on the surface of the substrate adjacent the at least one attachment region for detecting at least one of pH, oxygen, and calcium concentration of  
5 the medium.

24. The apparatus of any one of preceding claims 16-23, wherein the surface includes a plurality of attachment regions (96) which each attracts a single cell or a group of cells, each attachment region having an associated working electrode (88)  
10 positioned adjacent the attachment region.

25. The apparatus of any one of preceding claims 16-24, further including a retaining wall (56), formed on the substrate (70), for retaining the medium around the cell or cells.  
15

26. The apparatus of any one of preceding claims 16-24, further characterized by the substrate comprising a mesh, the medium disposed above and below the mesh.

20 27. A method of measuring efflux of a chemical from a biological cell (14, 52, 144, 216, 218), or a population of cells (170, 240, 242), the method including introducing the chemical to the cell and measuring an property of a medium (28, 152, 162, 172) surrounding the cell or population of cells, the property being related to a concentration of the chemical in the medium, the method characterized by:

25 positioning the cell or population of cells on a surface (74) of a substrate by attachment of the cell or the cell population to a region (72, 72', 126, 142) of the substrate which permits attachment, the attractive region of the substrate being surrounded by a region (78) which resists attachment of cells.

28. The method of claim 27, further characterized by:

- 48 -

30 the property being an electrochemical property and the step of measuring the property including employing a working electrode including a carbon ring electrode formed on the substrate adjacent the attractive region of the substrate.

29. A method of measuring transport of a chemical (26) across a membrane of a human or other biological cell (14, 52, 144, 216, 218), the method comprising exposing the cell to the chemical and measuring a property of a liquid medium (28, 152, 162, 172) disposed outside the cell, the property being related to a concentration  
5 of the chemical in the medium, the method characterized by:

providing a substrate surface (74) with a region (72, 72', 126, 142) formed from a material to which the cell attaches, the region being surrounded by a portion (78) of the surface which resists attachment of a cell;

10 patterning the substrate using photolithographic techniques to define at least one sensor (20, 50, 120, 160, 164, 180, 184, 188, 212, 214, 250, 122, 124) adjacent the attachment region for sensing the property of the liquid medium;

depositing the cell on the region; and

after the step of exposing the cell to the chemical, detecting the property of the liquid medium surrounding the cell and determining the concentration of the chemical in the medium therefrom.

30. A method of measuring transport of a chemical across a membrane of a biological cell (14, 52, 144, 216, 218), the method comprising exposing the cell to the chemical (26), the method characterized by:

5 providing a substrate surface (74) with a site formed from a material to which the cell attaches, the site being surrounded by a portion of the surface which resists attachment of a cell;

depositing the cell on the site;

moving a sensor (164, 212, 214) through a wall of the cell to contact the material in the cell; and

10 measuring a property of a material within the cell with the sensor, the property being related to a concentration of the chemical in the cell and determining the concentration of the chemical in the cell therefrom.

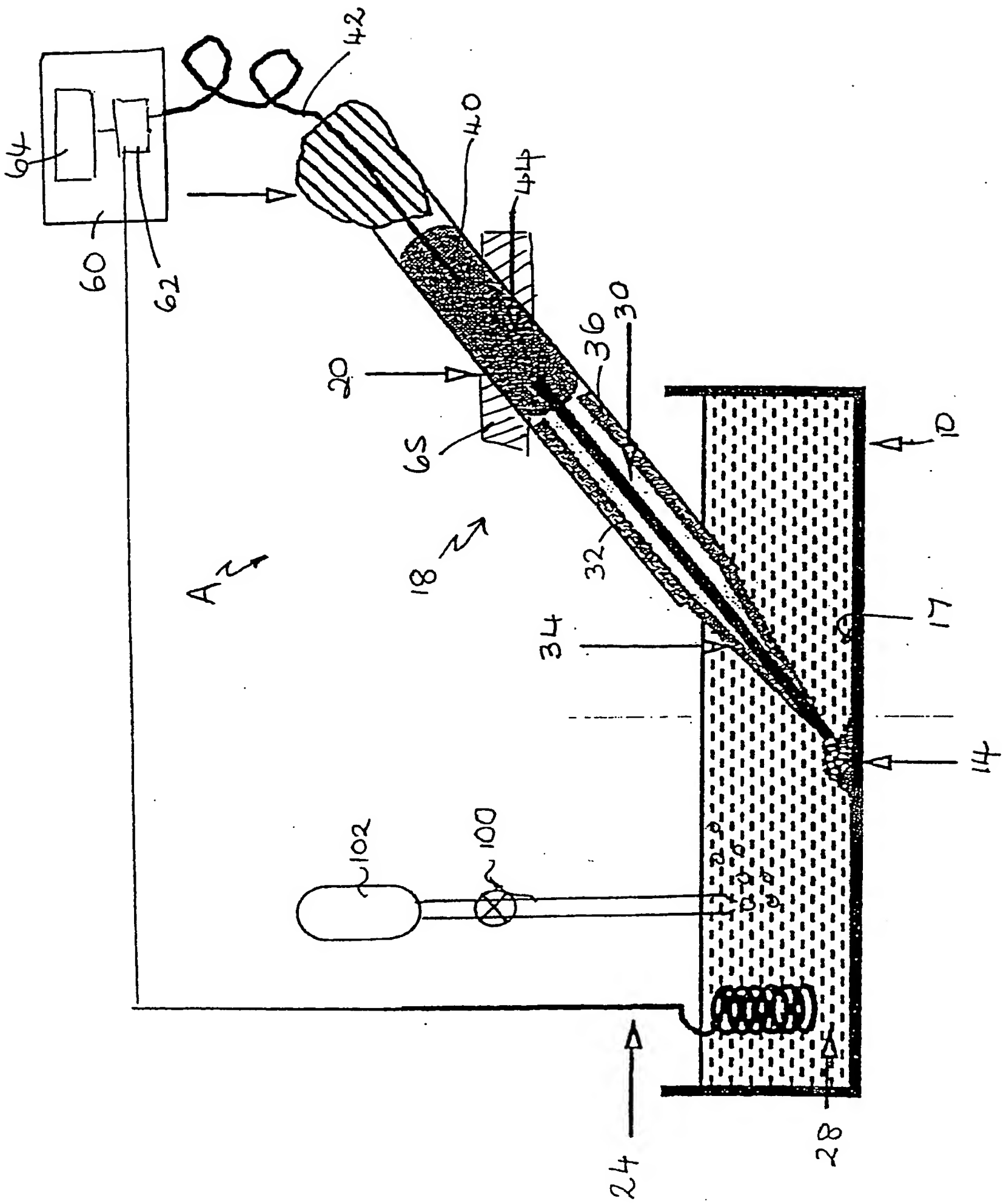


Figure 1



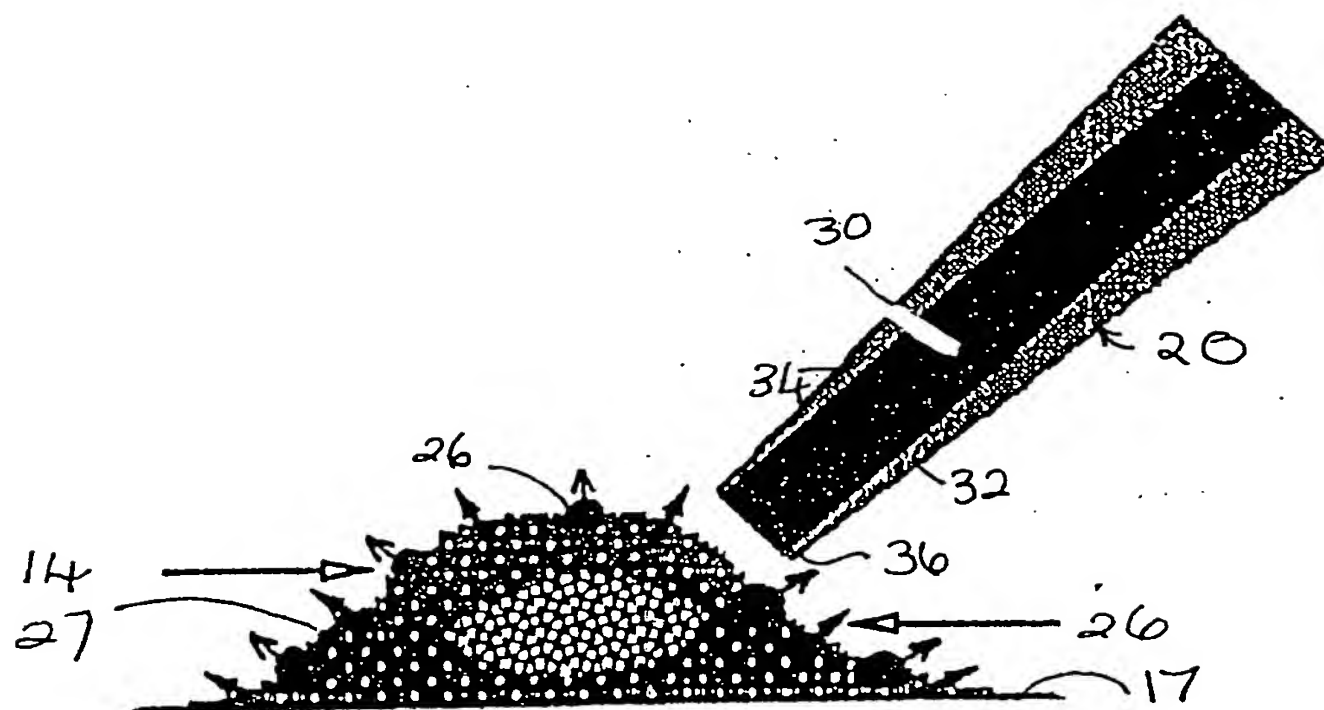


Figure 2

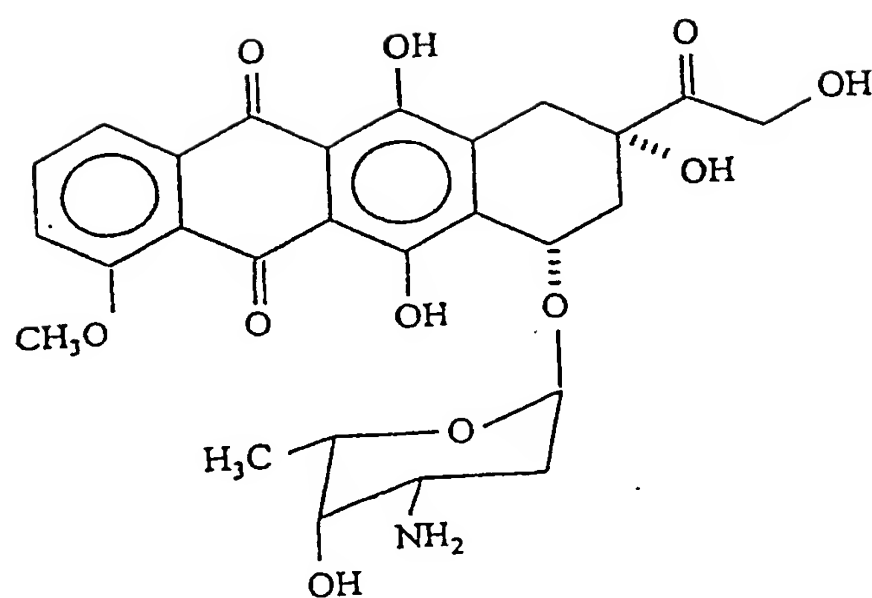


Figure 5

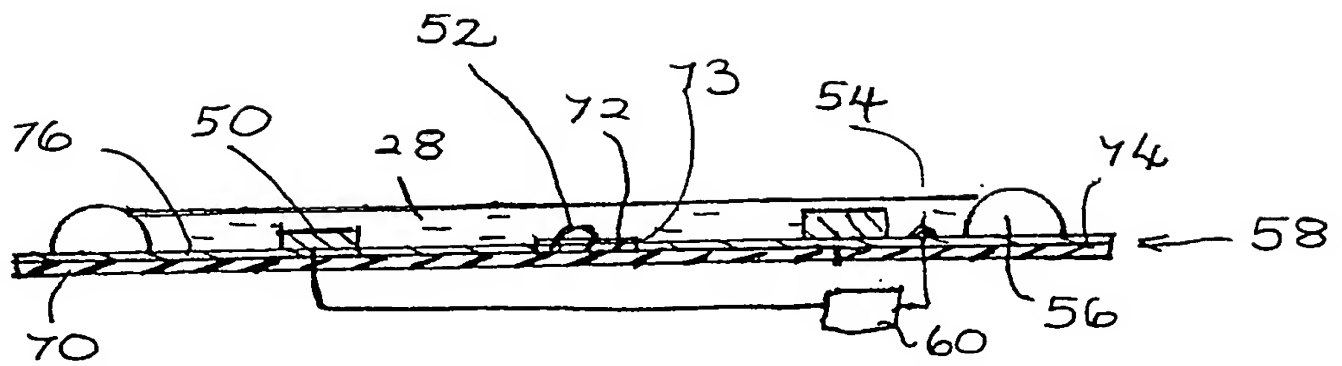


Figure 3

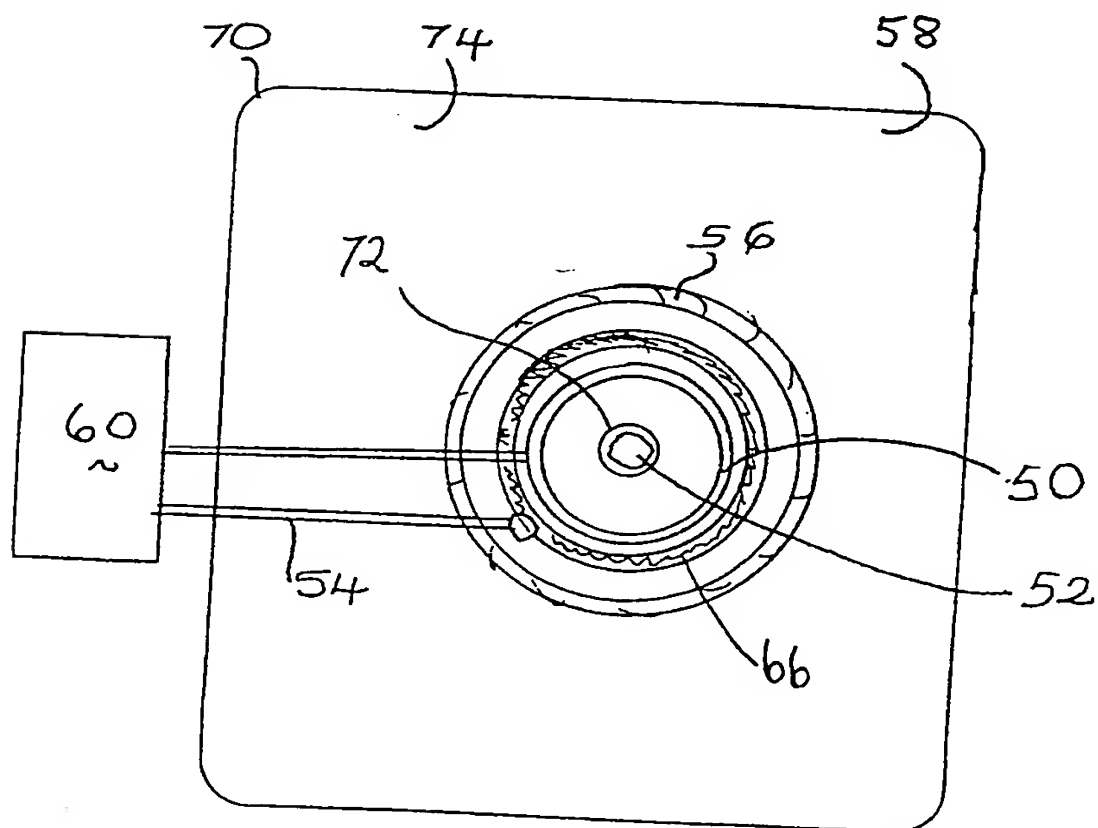


Figure 4

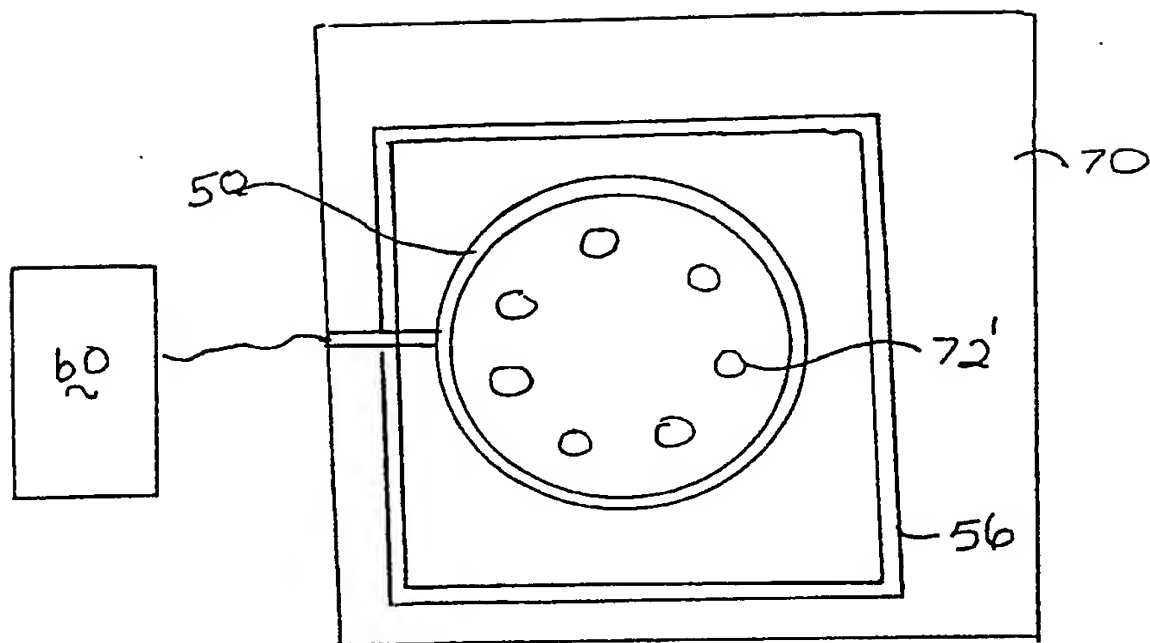


Figure 6

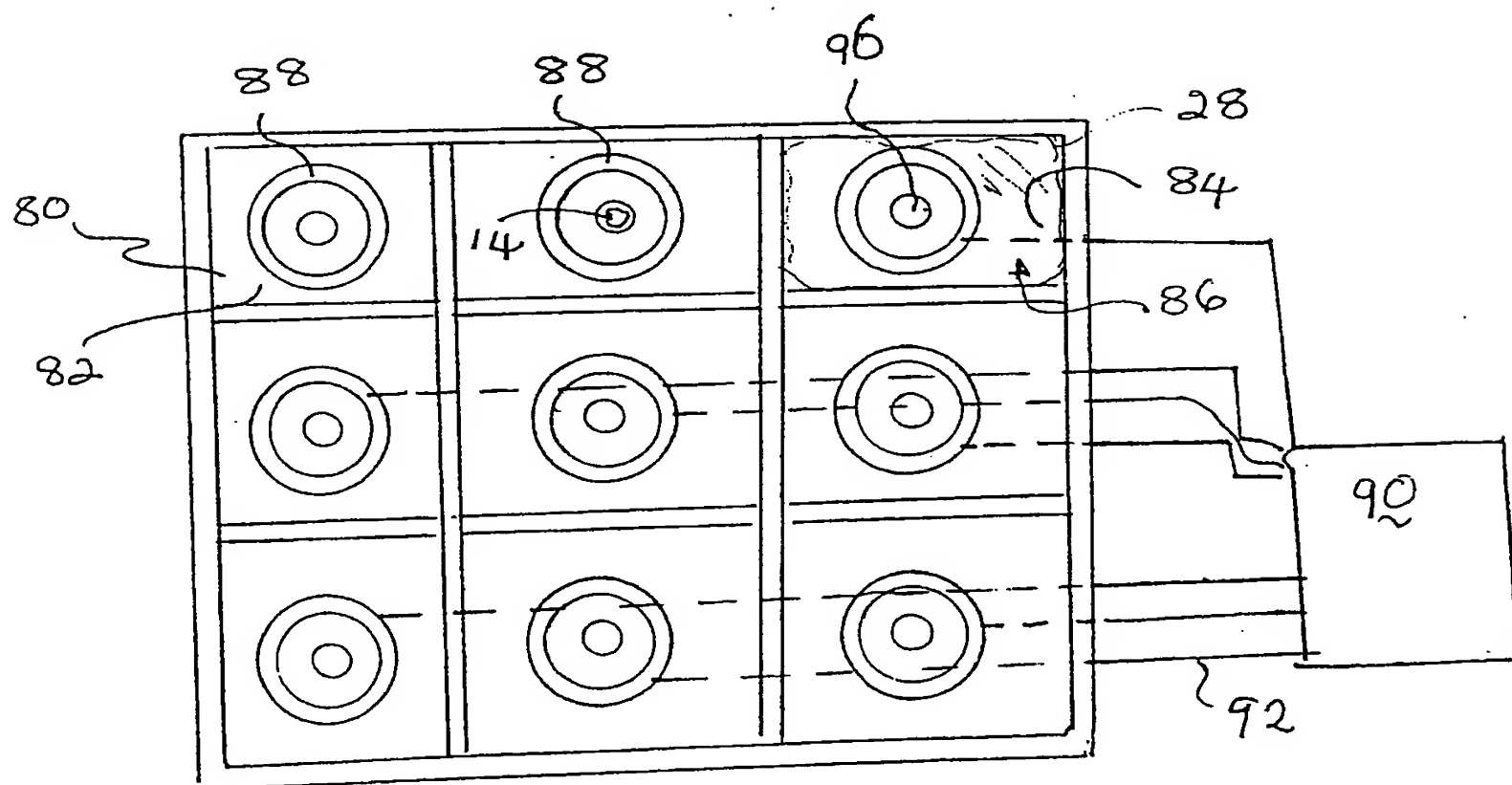


Figure 7

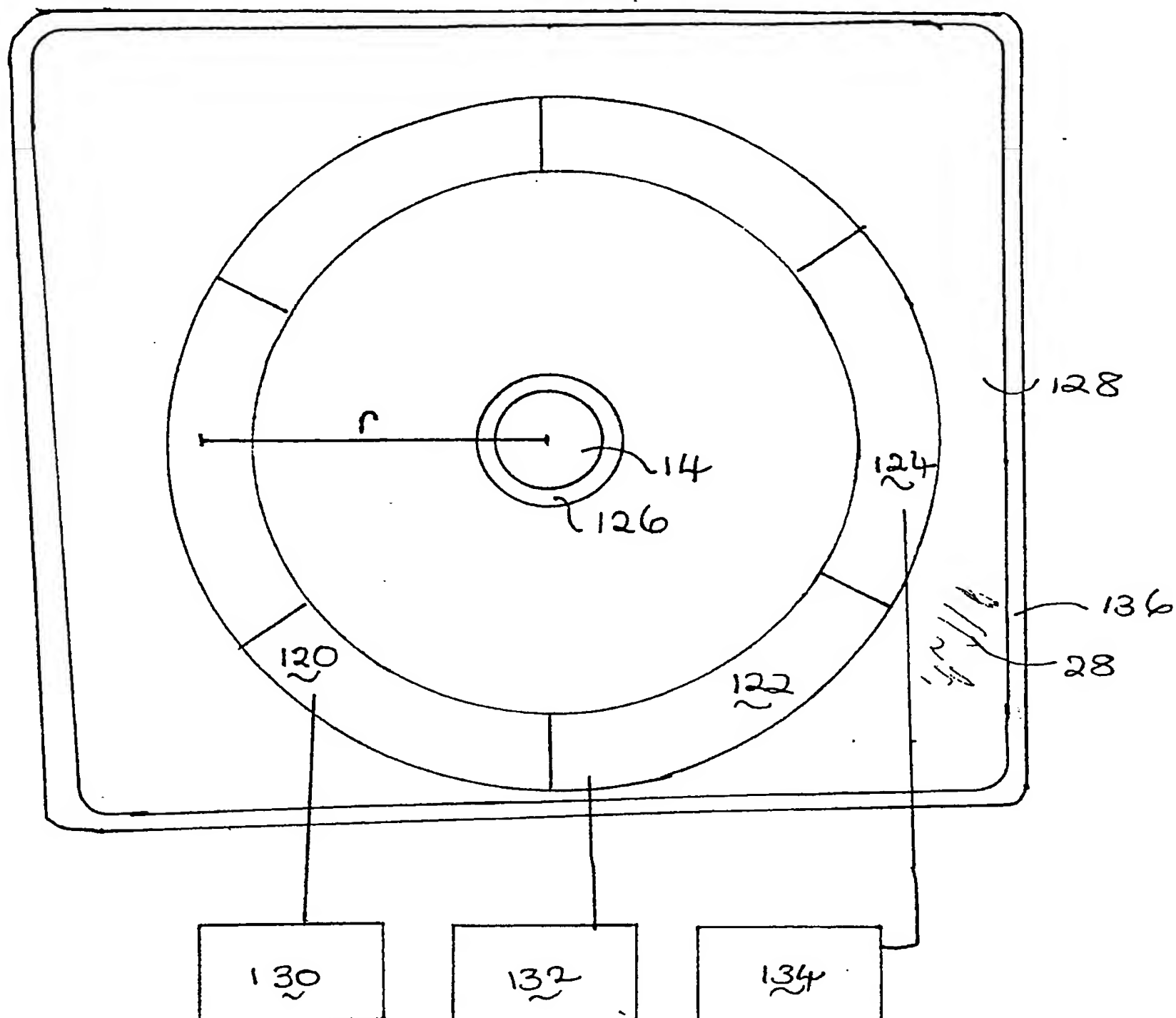


Figure 8

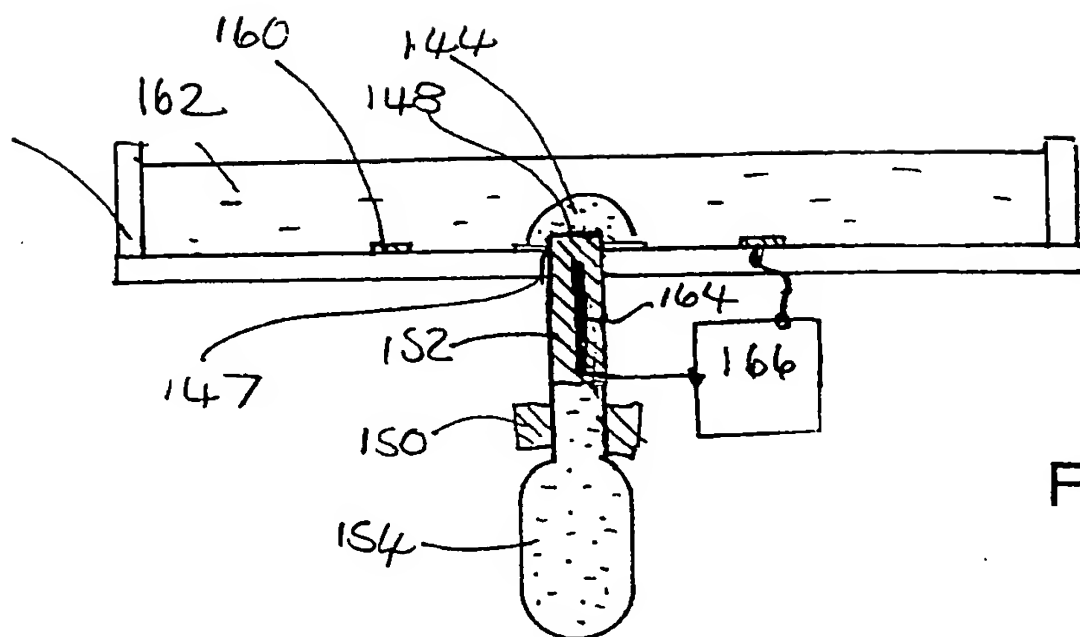


Figure 10

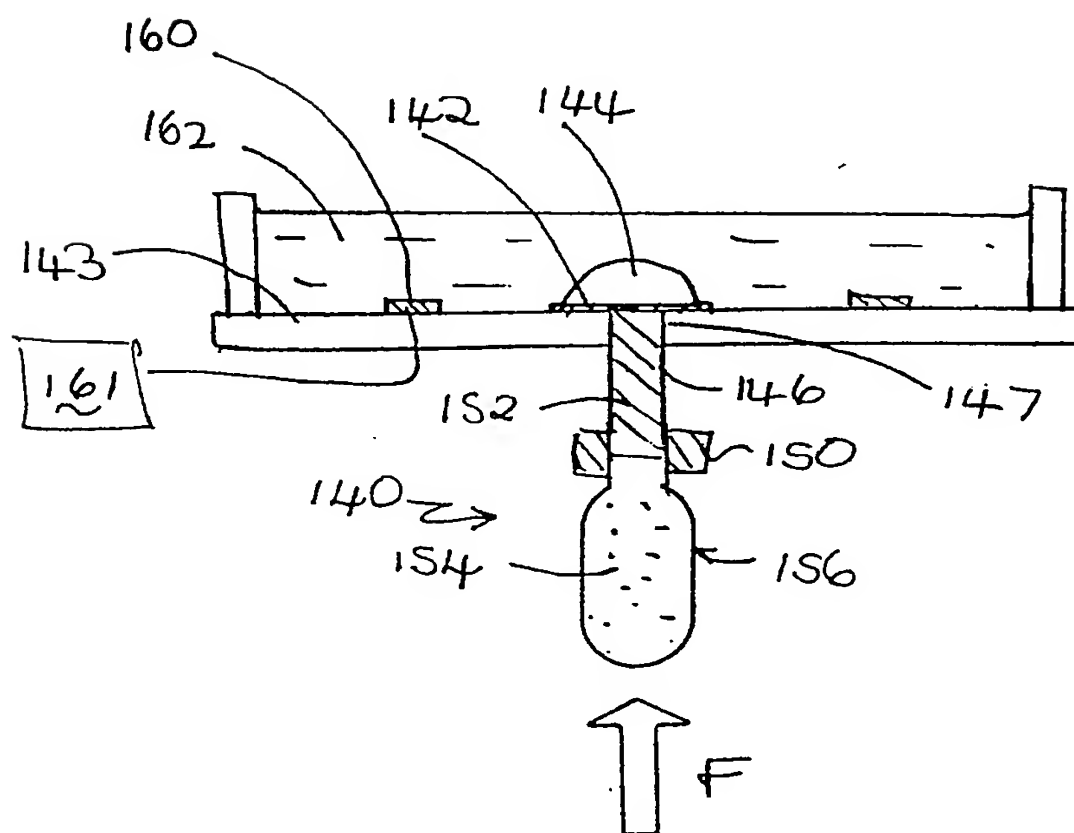


Figure 9

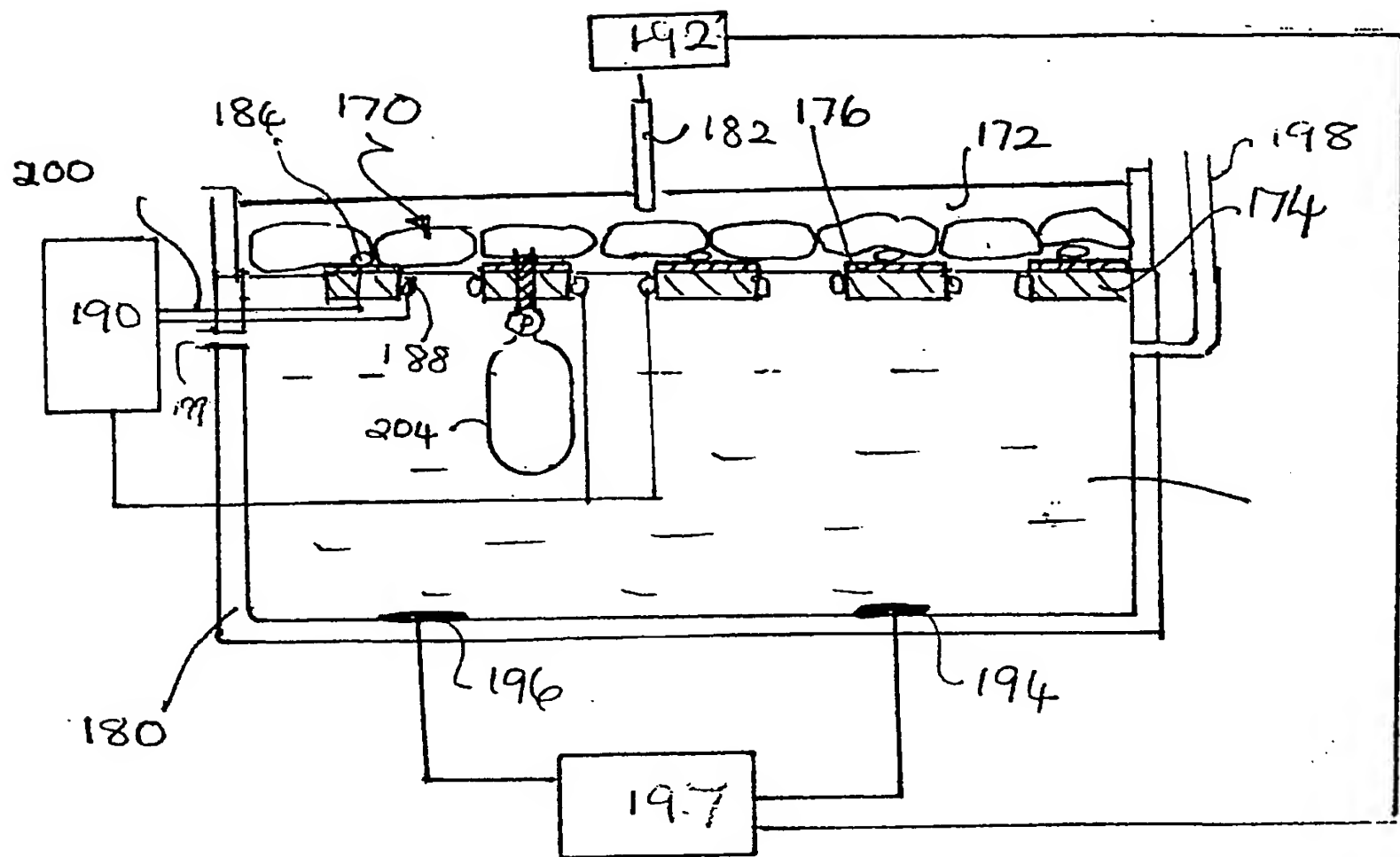


Figure 11

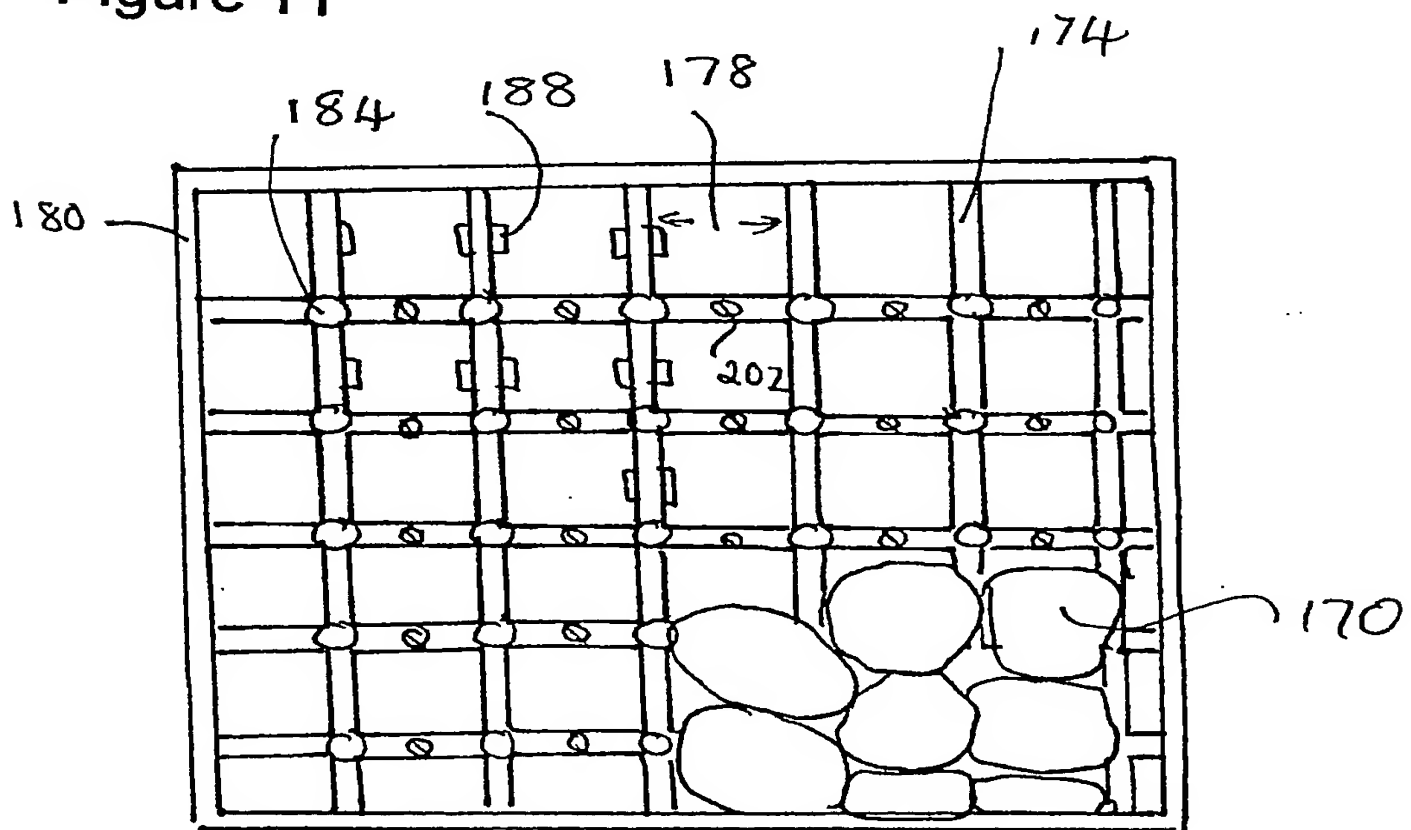


Figure 12

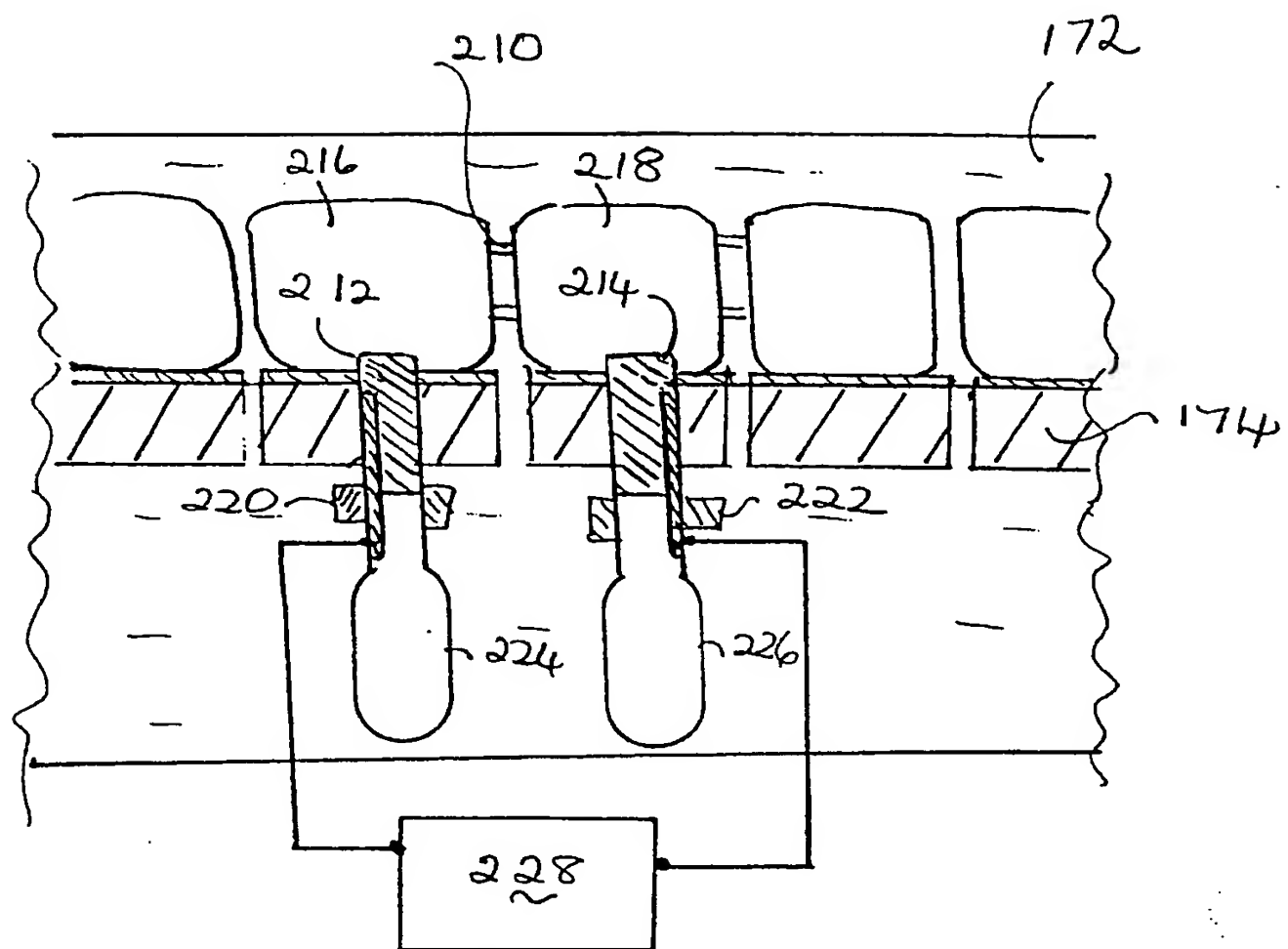


Figure 13

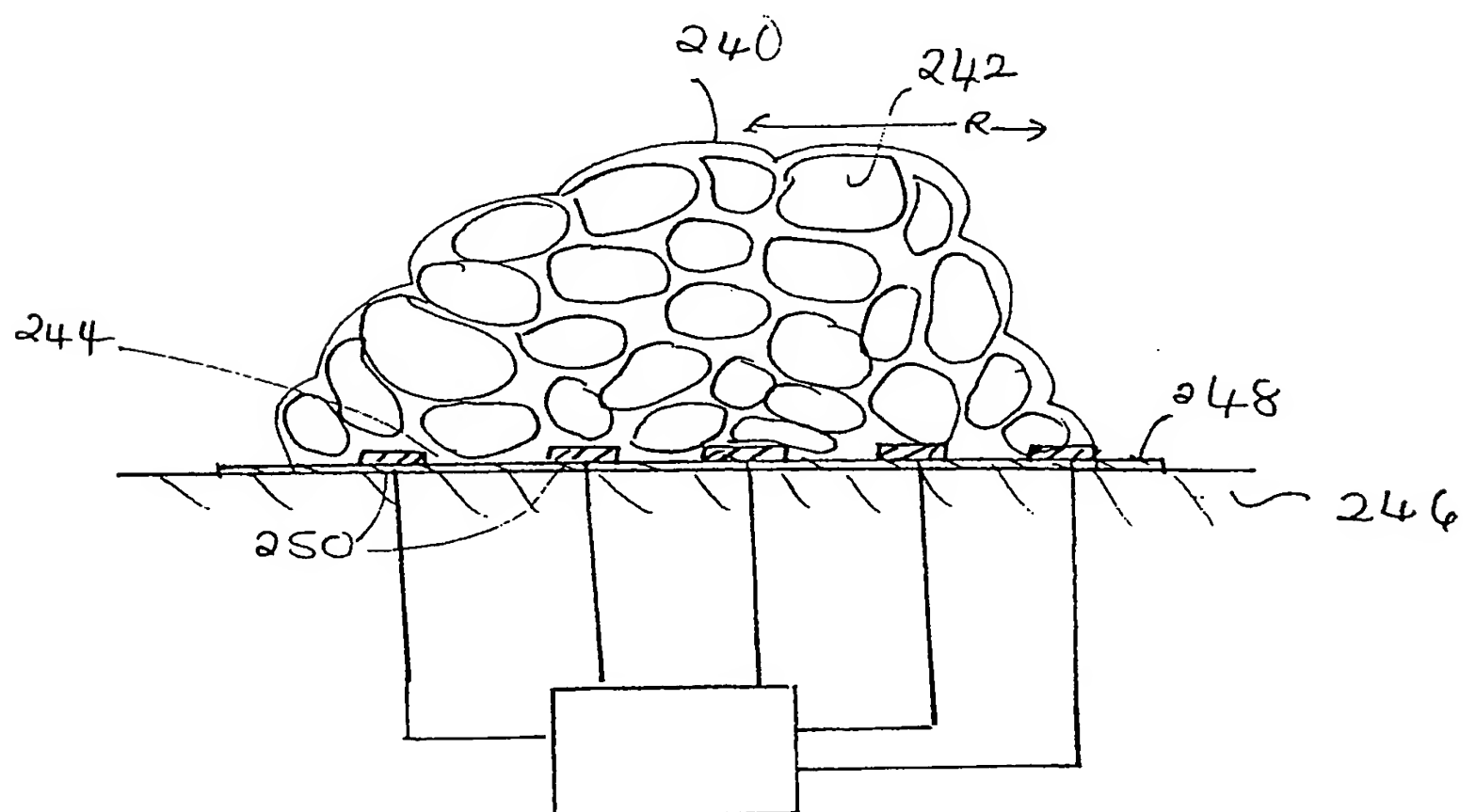


Figure 14



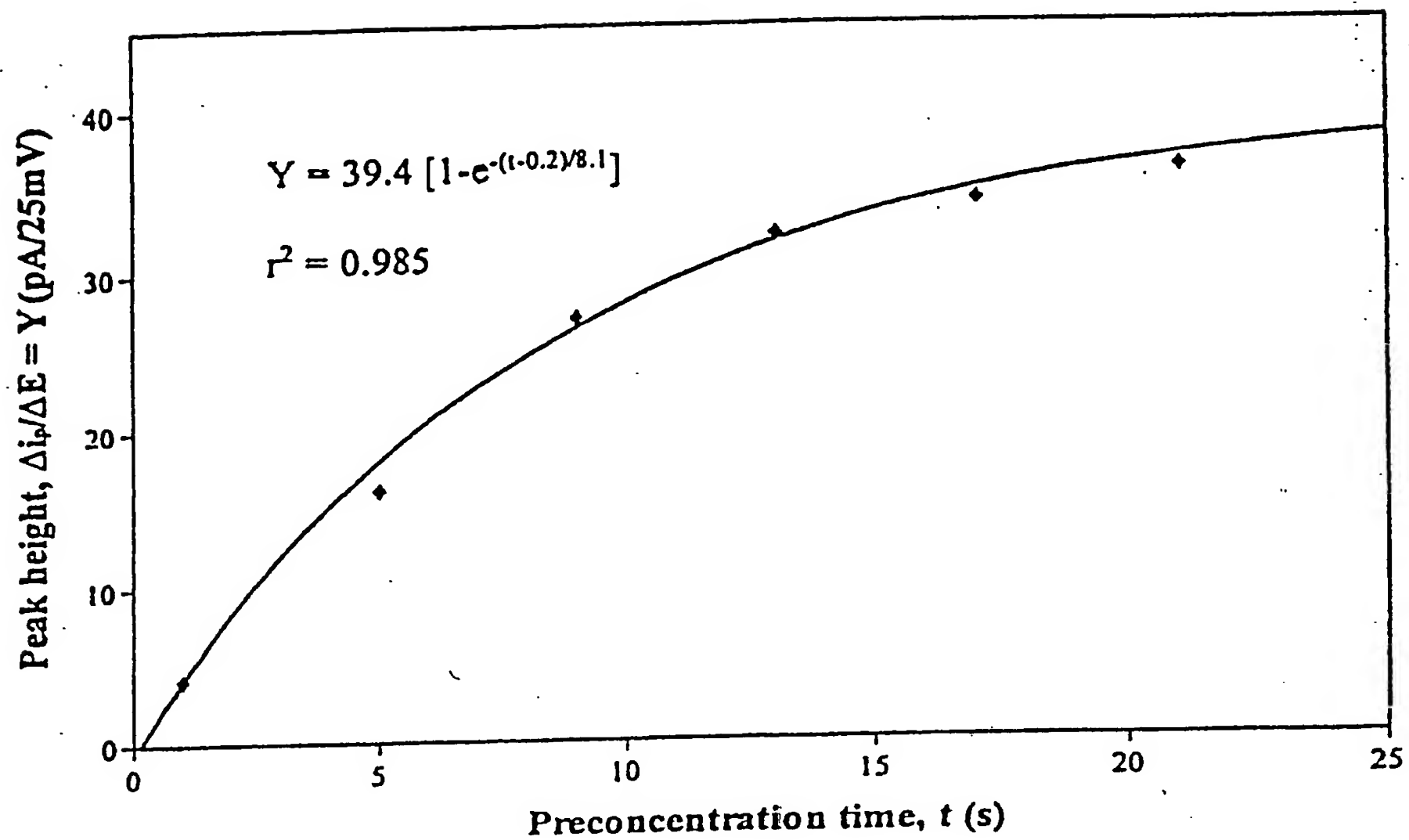


Figure 15

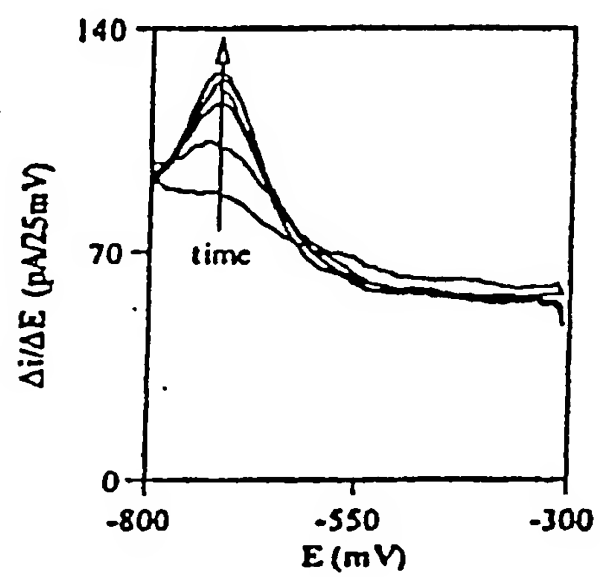


Figure 16

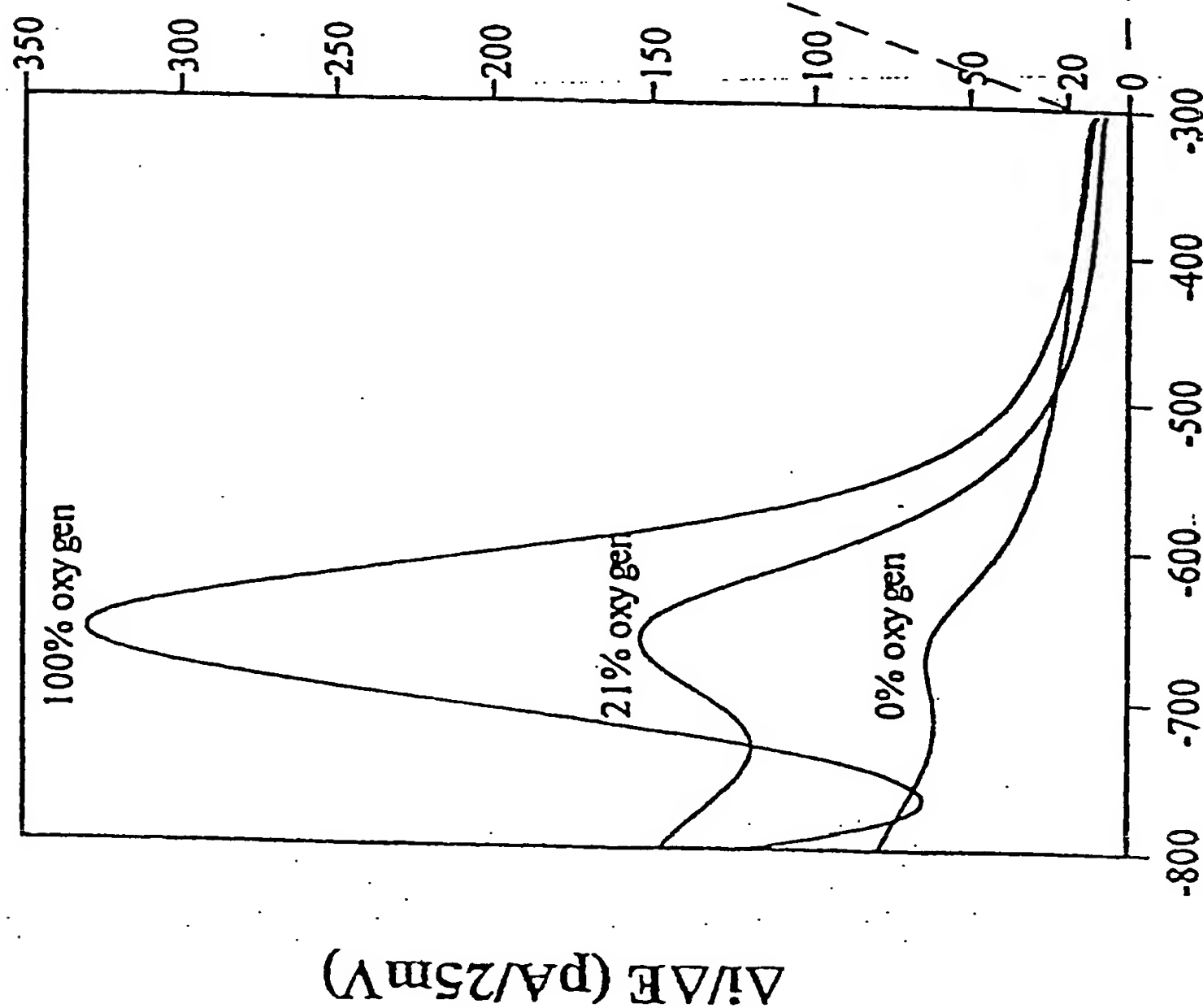


Figure 17

Potential,  $E$  (mV)

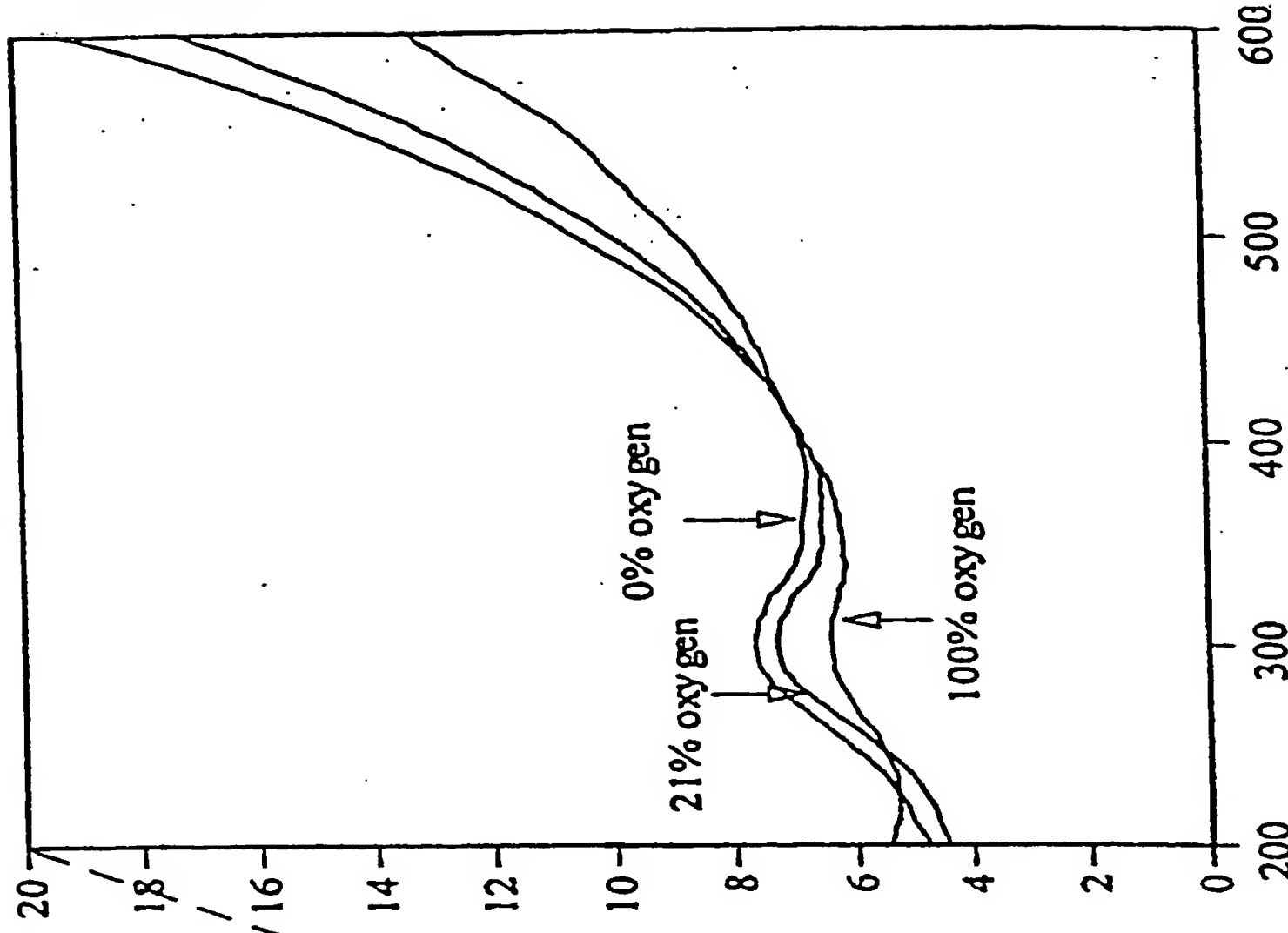


Figure 18

Potential,  $E$  (mV)

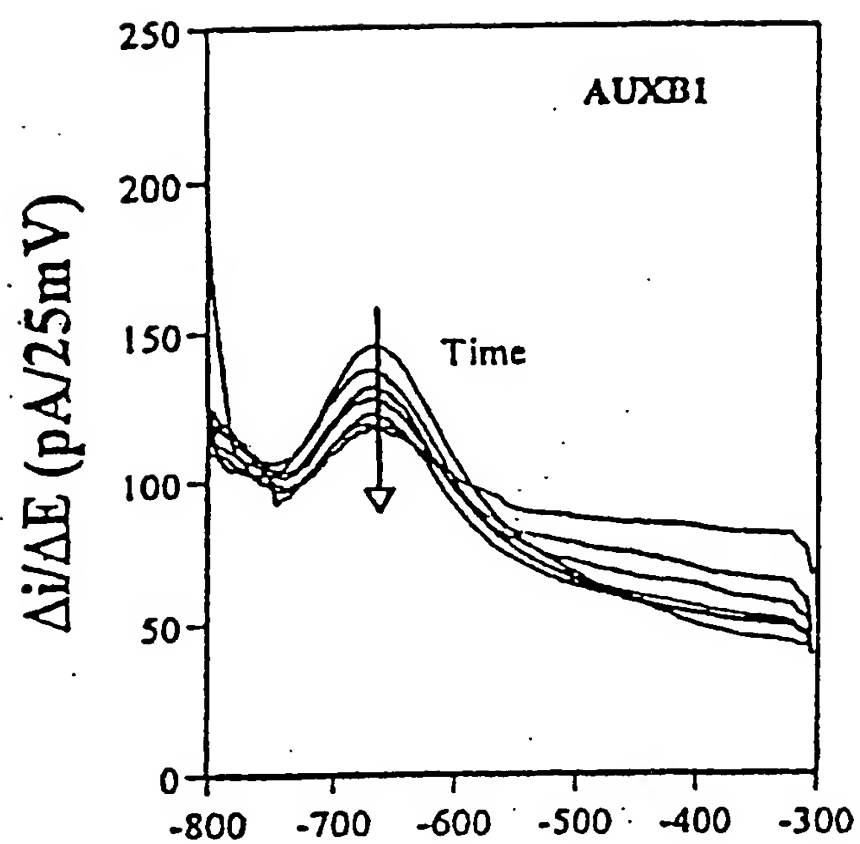
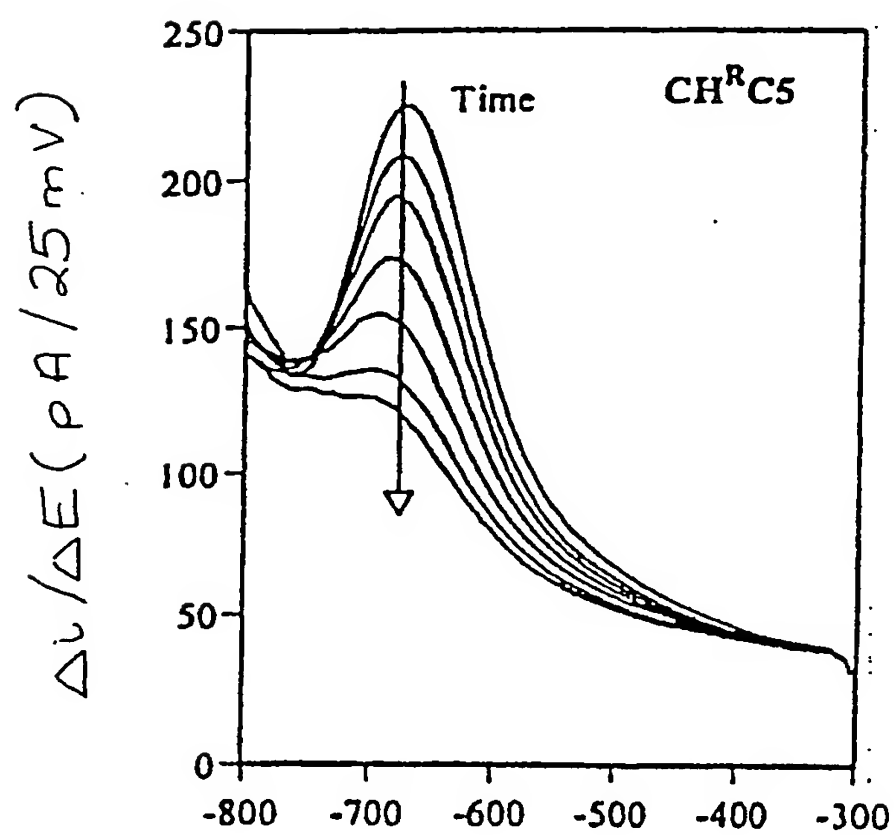


Figure 19



Potential, E (mV)

Figure 20

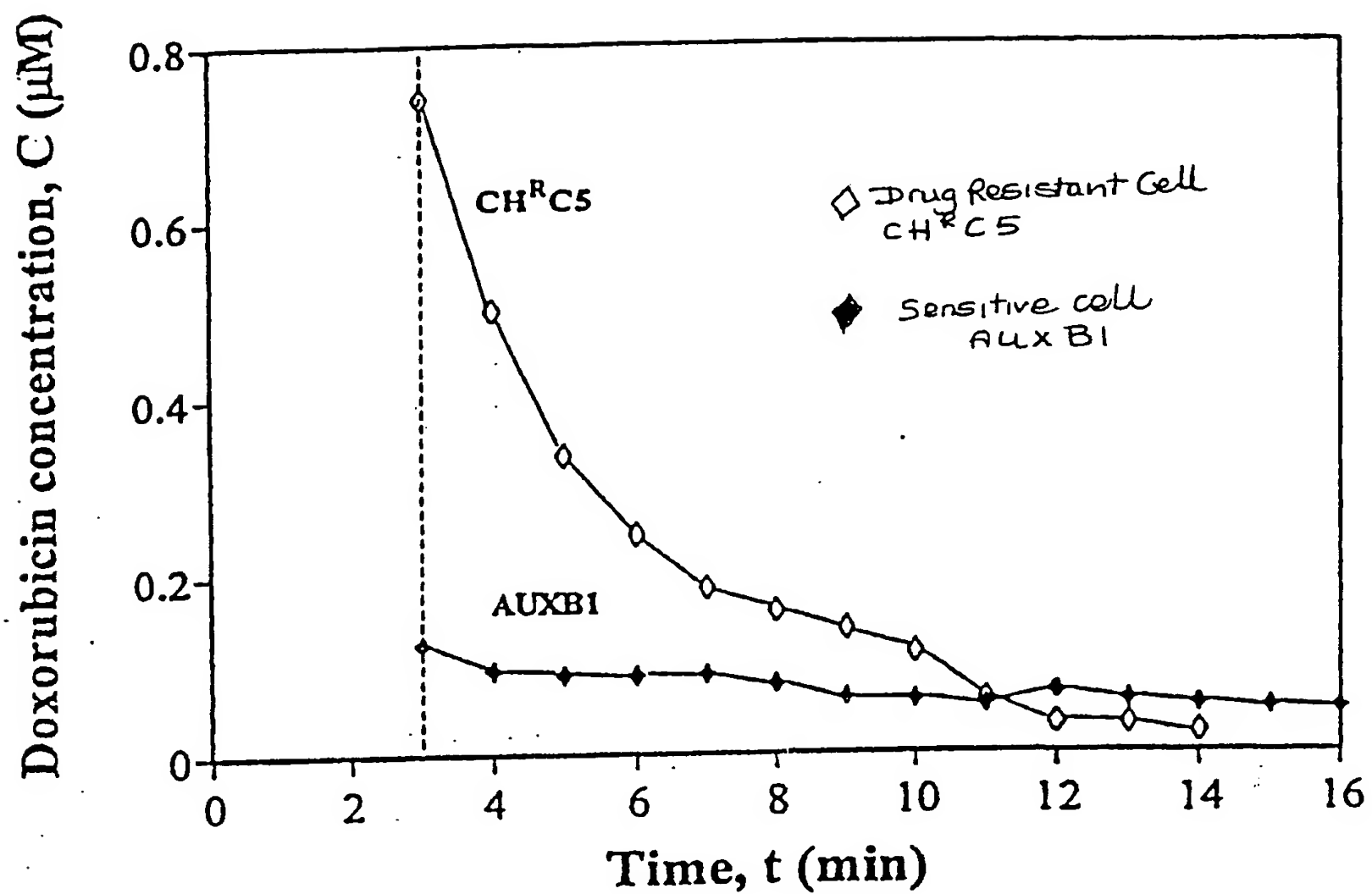


Figure 21

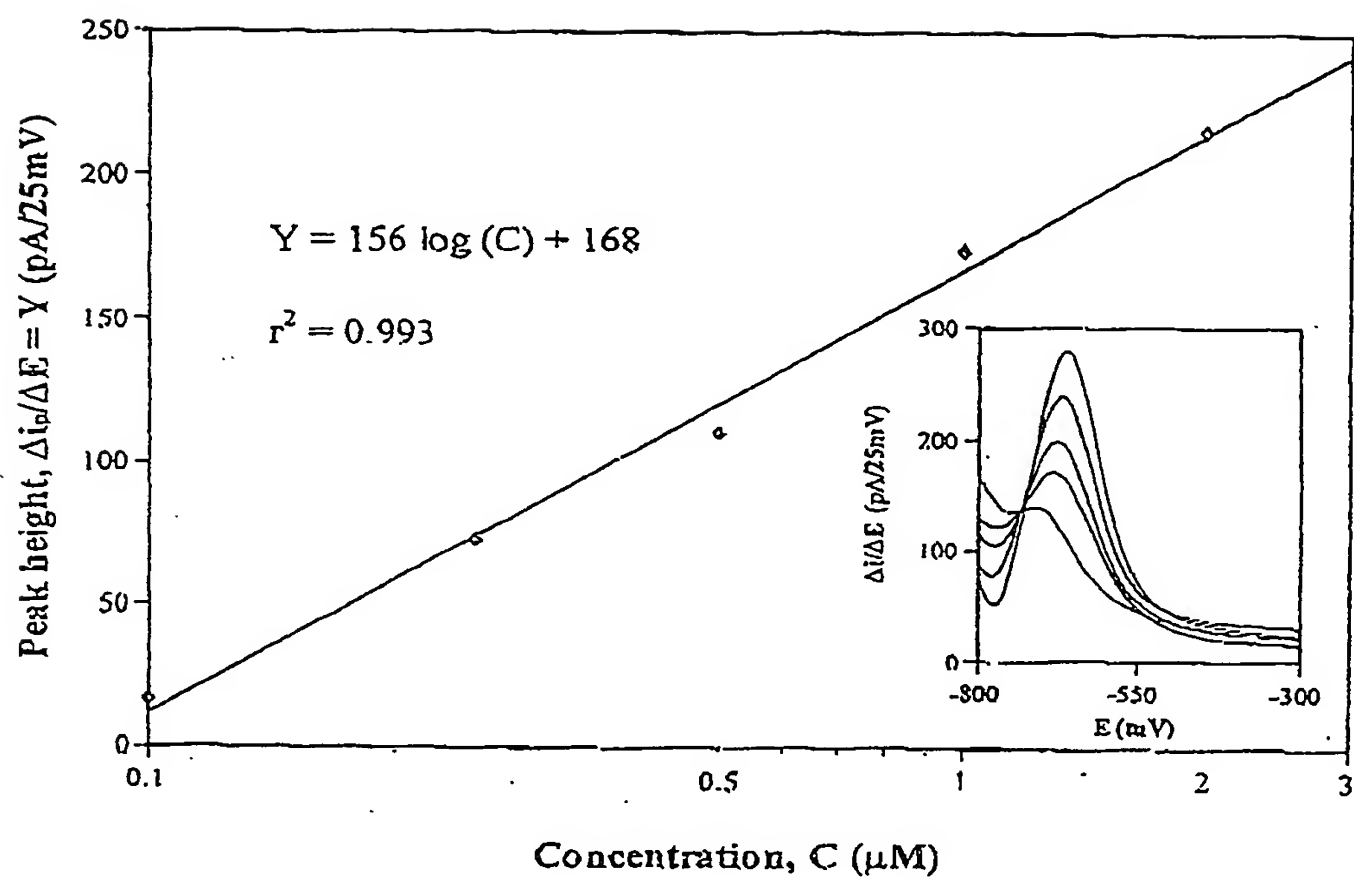


Figure 22